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STUDIES OF THE AFTER-RIPENING AND GERMINATION OF THE SEED OF *VIBURNUM TRILOBUM* MARSH.

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Studies of the After-ripening and Germination of the Seed of *Viburnum trilobum* Marsh." submitted by Paul Fedec in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

Seed of the American highbush cranberry *Viburnum trilobum* Marsh. maintained at 20° C required 328 days to reach total germination. Initially, a little more than half the seed was found to germinate within 140 days. After a 120 day lag, the balance of the seed germinated. After-ripening, with as little as 14 days of alternating temperature, shortened the time to total germination by four and one-half months.

The inductive effect of after-ripening was studied in relation to the utilization of the high lipid reserve present in the endosperm. No appreciable changes in polar and non-polar lipids occurred during prolonged exposure to either after-ripening or germinative conditions indicating that after-ripening is not associated with lipid degradation.

Fractionation of the water-soluble testa extract through Sephadex G-15 indicated the presence of four physiologically active materials, two having growth promoting properties and two behaving as inhibitors. Cumulatively, these materials show inhibitory activity when the crude extract is concentrated and stimulatory when it is diluted.

When seeds and embryos were treated with GA_3, there was no statistically significant effect on germination or embryo growth. However, when GA_3, at 100 ppm, was used on seed from which growth regulatory substances had not been removed, the effect of a significant interaction between GA_3 and the naturally occurring growth regulators on germination was noted. GA_3 was successfully used to overcome epicotyl dormancy of the seedling.

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INTRODUCTION

The American highbush cranberry *Viburnum trilobum* Marsh. (*V. opulus* var. *americanum* Ait.) is a deciduous shrub widely distributed throughout Canada from coast to coast and is quite common in the park-land region of Alberta. Its large clusters of white flowers, bright scarlet fruit and fall leaf color have caught the fancy of the general public and the plant is widely used as an ornamental shrub.

Dormancy is encountered in both seed and seedling of the high-bush cranberry during and subsequent to conditions generally prescribed for germination. The condition is characterized by slow growth of the radical and hypocotyl in some seeds and by what appears to be a complete reluctance to germinate on the part of others (Knowles and Zalik, 1958). Dormancy of the seed was found to be associated with the presence of a water-soluble inhibitor occurring in the seed coverings as well as a need for after-ripening. Dormancy of the seedling is expressed by failure of the epicotyl to grow following germination. Knowles and Zalik (1958) found that this type of dormancy could be overcome by simply removing the cotyledons.

Several questions concerned with the dormancy and germination of highbush cranberry were not answered in earlier studies and work was undertaken to look at some of these in greater depth. The effect of prolonged exposure to constant temperature on germination and germination processes had not been determined nor had the precise minimum requirement for after-ripening been ascertained. Though often used successfully to promote growth, the substance, gibberellic acid, had not been used on highbush cranberry seed to determine its effect as a germination promotor

nor had its effect on embryo growth or epicotyl dormancy been investigated.

Only preliminary investigations on the utilization of storage materials had been conducted and no attempt had been made to observe the response of excised embryos of the dormant seed to nutrient media. It had been noted that the endosperm of the seed was rich in lipid material, however, only cursory investigation of lipid utilization during after-ripening and germination had been undertaken. The presence of a water-soluble growth regulating material in seed coverings of high-bush cranberry had been noted but nothing had been done to determine its significance or, indeed, whether the material removed consisted of more than one component.

Because of the many unanswered questions, this study was undertaken with the following objectives in mind: (1) to determine the significance of after-ripening treatment to total germination and in the utilization of lipid material, (2) to investigate the nature of the water-soluble growth regulatory material existing in seed coverings and (3) to determine the effect of exogenous materials on growth and development of dormant embryos, seeds and seedlings.

LITERATURE REVIEW

Seed germination can be regarded as a series of steps which cause a quiescent seed with a low water content to show a rise in metabolic activity that culminates in development of a seedling (Mayer and Poljakoff-Mayber, 1963). For a seed to germinate, it must be provided with suitable environmental conditions. Required is an adequate supply of moisture, a favorable temperature and an adequate supply of oxygen. Even when these necessary conditions are provided, it does not follow that the seed will germinate in all cases. Such a seed is said to be dormant.

Amen (1968) considers a seed as dormant "when and if some inherent condition precludes further development unless a special agent is supplied to the dormant system. This inherent condition may involve active or passive inhibition or impermeability resulting in partial metabolic arrest."

Dormant seeds may possess: (1) rudimentary embryos, (2) physiologically immature embryos, (3) mechanically resistant seed coverings, (4) impermeable seed coats, or (5) substances which inhibit germination. The dormancy of a particular seed may be attributed to one or to a combination of these factors.

The dormancy of its seeds for an extended period of time following harvest is of great importance in the ontogeny of a plant. Without dormancy a species could conceivably fail to survive unfavorable conditions. For example, seeds of a large number of woody species of temperate climates ripen and are shed in the fall. Though moisture and

temperature are often suitable at that time, the seeds do not germinate. If they did, the seedlings might not survive the winter that follows. A prerequisite to germination of this type of seed appears to be a period of after-ripening.

After-ripening results in a series of changes in the seeds that lead to improved germination. After-ripening can occur in dry storage in some seeds, however, it more frequently occurs in fully imbibed seeds when these are held at temperatures between 2 and 5° C for varied periods of time. It is an interesting phenomenon but, unlike most physiological processes, does not follow Van't Hof's principle that the rate of a reaction increases with an increase in temperature. Commenting on the changes which occur during after-ripening at low temperature, Stokes (1965) stated that the types of changes are balanced in such a way that they are able to proceed most rapidly at temperatures slightly above freezing.

During after-ripening the composition of the storage materials present in the seed may alter, for example, in cow parsnip (*Heracleum sphondylium* L.) acceleration of amino acid formation from reserve protein occurs (Stokes, 1953b). As a result of after-ripening, permeability of the seed coat may change as in the case of cocklebur (*Xanthium canadense* Mill.) where after-ripening alters permeability of the testa to oxygen (Thornton, 1935). Substances promoting germination may appear or inhibitory ones disappear during after-ripening, for example, in the seed of the hazel-nut (*Corylus avellana* L.) after-ripening brings about synthesis of gibberellin, a germination promoting substance (Bradbeer, 1968). After-ripening may also bring about growth and developmental changes

in immature embryos of some seeds, for instance, immature cow parsnip embryos comprise 0.4% of the dry weight of the resting seeds. However, at the end of the after-ripening period, the fully developed embryos constitute 30% of the weight of the seed (Stokes, 1965).

Localization of the dormant condition in a specific part of the embryo is seen most strikingly in seedlings of lily, *Lilium* sp. (Barton, 1936); tree peony, *Paeonia suffruticosa* Haw. (Barton and Chandler, 1957) and highbush cranberry, *Viburnum* sp. (Giersbach, 1937). In such cases the root emerges from the seed without the usual exposure to low temperature, however, the epicotylar portion of the seedling does not grow. Epicotyl dormancy is generally overcome by subjecting the seedling to two or three months of low temperature (2 - 5° C).

Biochemical Processes in Germinating Seeds

The biochemical processes of germination begin with imbibition. To a great degree this process is influenced by the colloidal nature of the materials within the seed. During imbibition, seed colloids are hydrated, swelling occurs and as a result of internal pressure, the seed coat may be broken open. Imbibition, however, is not dependent on viability (Mayer and Poljakoff-Mayber, 1963).

In viable seeds, hydration of the tissues is generally accompanied by the activation of enzymes, an increase in the respiration rate and a breakdown of stored materials. These later serve as substrates for respiration and at the same time provide raw materials required for growth of the embryo.

Seeds can be divided into those in which the main storage material is carbohydrate and those in which the main storage material is lipid. Lipid-containing seeds are the more numerous of these two groups (Mayer and Poljakoff-Mayber, 1963), though economically important seeds are mostly of the former type. The protein content of either seed group may vary from 3 to 35 per cent, however, few seeds have protein as the predominant storage material. Seeds generally high in lipid (e.g. flax, *Linum usitatissimum* L., 34% and rape, *Brassica rapa* L., 40%) tend also to have high amounts of protein (23 and 25% respectively, Mayer and Poljakoff-Mayber, 1963). Knowles (1957) reported the seed of American highbush cranberry (*Viburnum trilobum* Marsh.) to contain 25.17% lipid and 3.89% total nitrogen. Assuming a conversion factor of 6.25 for nitrogen to protein, the estimated protein in highbush cranberry would be 24.3%.

The two chief forms in which carbohydrates are stored in seeds are starch and hemicellulose. These are broken down by amylases and glucosidases to glucose, which is utilized by the embryo for growth and development.

According to St. Angelo and Altschul (1964) "seeds of high lipid content metabolize most of their stored lipids within a week after the start of germination." The first step in the conversion is the hydrolysis of triglycerides to fatty acids and glycerol (Varner, 1965; Zimmerman and Klosterman, 1965). This is accompanied by increased neutral lipase activity. The lipase either exists in the resting seed as an active enzyme or precursor or is synthesized at the time of germination (St. Angelo and Altschul, 1964).

The free fatty acids do not accumulate in the seed but are converted by the β-oxidation process to Acetyl-Coenzyme A. This enters into the glyoxylate cycle where it is converted to phosphoenolpyruvate. The phosphoenolpyruvate, after passing through the glycolytic cycle, ends up as sugar for utilization by the embryo (Stumpf, 1962; Robinson, 1967).

Malate synthetase and isocitratase, enzymes of the glyoxylate cycle, are present in oil-bearing seeds in minute amounts or are absent until several days after the start of germination. It is thought that malate synthetase and isocitratase are synthesized at the time when lipid degradation is observed. The synthesis of these enzymes may be in response to some hormone produced in the root-shoot axis concurrent with the need for growth substrate (Varner, 1965).

According to Zelitch, the enzymes malate synthetase and isocitratase are not found in germinating seeds of cereals and legumes, seeds which are low in lipid reserve (Schramm, 1967). Studying a number of germinating coniferous seeds, Firenzuoli *et al.* (1968) noted that where lipid was present, enzymes of the glyoxylate cycle were also noted.

Of the total lipid fraction present in seed, the polar phospholipids are of some importance. Phospholipids do not appear to function as a reserve substrate (Singh, 1967) rather, they are universal components of cell membranes and function in the control of cell permeability (Conn and Stumpf, 1967). Phospholipids have also been found to play a role in electron transfer in mitochondria (Ansell and Hawthorne, 1964; Green and Kopaczyk, 1966) and in protein synthesis (Ansell and Hawthorne, 1964),

hence it is not difficult to picture an 'essential' role for phospholipids in germination.

Redshaw and Zalik (1968) observed an inverse relationship between the levels of polar, mainly phospholipids, and non-polar lipids, mainly triglycerides, in seeds during vernalization of Sangaste fall rye (*Secale cereale* L.) and Kharkov winter wheat (*Triticum vulgare* L.). The level of the polar lipid fraction in the seed of fall rye increased from 29.4% in ungerminated seed to 42.6% by the time the seedlings were six weeks old. The polar lipids of winter wheat increased from 29.3% initially to 45.9% at six weeks. Concomitant with the increases in polar lipids of the two crops were proportional decreases in non-polar lipids. These findings suggest the synthesis of polar lipids from non-polar lipids in early phases of germination. Zimmerman and Klosterman (1965), working with flax, noted increased amounts of phospholipids when non-polar lipids dropped to 47% of the original amount after 90 hours of germination. They also noted that the fatty acid composition of the phospholipids in resting flax seed was different from that of the young seedling.

During germination, enzymatic breakdown of reserve protein in seeds occurs with a concomitant increase in amino acids (Varner, 1965; Splittstoesser, 1969a; Wang, 1968). Some of these amino acids are oxidatively deaminated and the carbon skeleton enters the Kreb's cycle. The ammonia thus formed is converted to amides and thus rendered harmless to plant tissues; the chief amides formed being glutamine and asparagine. Other amino acids are utilized as nitrogen sources for the synthesis of new proteins and nucleic acids in the actively growing embryo (Splittstoesser, 1969a).

Temperature Effects on Seed Dormancy

In dormant seeds, the optimum temperature for germination is not often the same as the optimum temperature for after-ripening. After-ripening temperatures are frequently lower than those at which germination takes place. Indeed, it has been shown that some seed will after-ripen more readily at alternating high and low temperatures.

Stokes (1965) noted that low temperature after-ripening of some oil rich seeds, for example, hawthorn (*Crataegus* sp.), juniper (*Juniperus* sp.) and maple (*Acer* sp.), is associated with a dispersal and sometimes disappearance of lipid storage materials. It was suggested that these lipid materials, present in the surface layers of the cell protoplast of dormant seeds, may play an important part in dormancy by regulating the water permeability of the cell membranes. Accumulation of lipid material in the membrane may impose dormancy in seed by restricting imbibition. Upon dispersal of the lipid, the membranes become permeable and imbibition can occur.

Seed reserves of hawthorn (Singh, 1967), maple, juniper and cow parsnip consist mainly of lipid and protein and have little carbohydrate (Stokes, 1965). During after-ripening, increased acidity, water holding capacity, catalase and peroxidase activity have been observed in these seeds. At the same time, increases in reducing sugar content and in rate of respiration have been measured.

Germination of cow parsnip seed at 20° C after low temperature after-ripening at 2 - 5° C was attributed to the diffusion of some unknown product of low temperature embryo metabolism into the endosperm.

This product, acidic in nature, it is suggested, functions in the breakdown of storage protein during germination after a critical level of product has been established during after-ripening (Stokes, 1953a). Stokes later believed that embryo growth in cow parsnip seed at 20° C was prevented by nitrogen starvation of the embryo. Stokes (1953b) found that low temperature after-ripening of cow parsnip seed not only increased hydrolysis of protein in the endosperm but brought about the accumulation of those amino acids needed for embryo growth, namely, arginine and glycine. These were not accumulated in great enough quantities for embryo growth at higher temperatures.

Singh (1967) has noted that some of the chemical changes effected by low temperature after-ripening of seed are increased enzymatic activity, increased accumulation of sugars and amino acids and increased transformation of insoluble nitrogenous substances into soluble proteins and amino acids.

In the case of the dormant epicotyl of tree peony, Fine and Barton (1958) stated that synthesis of proteins in the region of the epicotyl cannot occur without the movement of amino acids from the endosperm where they are stored as protein or as free amino acids. When the seedling is subjected to low temperature after-ripening, this facilitates proteolysis followed by transfer of amino acids to the epicotylar region. Results from epicotyl dormancy studies in highbush cranberry seedlings (Knowles and Zalik, 1958) appear contrary to Fine and Barton's proposal. Knowles and Zalik were able to overcome the dormancy of the epicotyl in highbush cranberry seedlings simply by excising the cotyledons. This procedure effectively by-passed the need for low temperature after-ripening.

Alternating temperatures have often been successfully employed as a method of after-ripening some dormant seeds. In nature, seeds in the soil undergo a daily alternation of temperature ranging from warm in the daytime to cool at night. The mechanism behind the favorable effect induced by alternating temperature has not been conclusively established. However, it has been suggested that a heat sensitive, complex macromolecular compound (such as an enzyme precursor) may be sufficiently modified by changes in temperature to permit after-ripening or that internal daily rhythms may exist in seed. When cycles in the component parts of the seed are out of phase with each other, alternating temperatures may assist to bring the phases into step (Koller *et al.*, 1962).

Amen (1968) in proposing a model for seed dormancy considered temperature as a possible trigger mechanism for germination. Though he felt that it was difficult to reconcile temperature effects with after-ripening, there was, however, a possibility that different enzyme systems might be activated by different temperature treatments.

Growth Regulators as Factors Affecting Germination

A. Growth inhibitors

Failure of certain seeds to germinate immediately after harvest may be due to the presence of inhibitory substances in the pericarp, endosperm or seed coat. Many naturally occurring substances inhibit germination and it is quite possible that any substance, when present in sufficiently high concentration, can inhibit growth (Wareing, 1966).

These inhibiting substances are often non-specific in their action (Barton, 1965).

According to Wareing (1966) inhibitors to growth or germination of bioassay material can almost always be detected in extracts from seeds showing dormancy. Wareing further stated that the diverse chemical nature of the substances shown to have inhibitory properties in simple tests makes it unlikely that they all function as growth regulators.

There tends to appear in the literature inconsistencies in terminology with reference to substances showing growth regulatory activity. Though no official definition of growth inhibitor has been adopted, Larsen (Mer, 1968) suggests the term be applied to substances which retard cell enlargement in both shoot and root cells and have no stimulatory range of concentration. If a substance promotes germination or cell enlargement at some other concentration, it should correctly be termed a growth regulator. "Hence, under the action of an inhibitor, growth would be depressed as compared with untreated controls" (Mer, 1968).

Extracts of seeds and seed coverings have frequently been studied to determine presence of substances inhibitory to germination. Knowles and Zalik (1958) in studying extracts from the testa of highbush cranberry noted the presence of water soluble substances that inhibited germination. In addition to this, water extracts of the endocarp inhibited root growth of wheat and highbush cranberry. Corns and Schraa (1962) found similar indications of inhibitory substances in water extracts of the endocarp of the fruit of Silverberry (*Elaeagnus commutata* Bernh.). Extracts of the endocarp were found to retard growth of wheat

seedlings and removal of the endocarp enhanced germination of Silverberry seed.

Abscisic acid has been found in high concentration in the seed coats of deeply dormant seeds of Ayrshire rose (*Rosa arvensis* Huds.) by Cornforth *et al.* (1966). It was suggested by Bradbeer (1968) that abscisic acid may be responsible for its dormancy. It had earlier been reported that phenolic substances were responsible for the dormancy of Ayrshire rose (Jackson and Blundell, 1965). This would suggest, perhaps, that two inhibiting substances are associated with the dormancy of this particular seed.

Bradbeer (1968) has attributed dormancy of Kent cob hazel-nuts (*Corylus avellana* L.) to the presence of inhibitors in the seed coat. The seed coat and endocarp of hazel-nut have been shown to contain a number of unidentified substances which are inhibitory to the growth of wheat embryos.

Daletskaya (1964) found concentrations of 1×10^{-4} to 1×10^{-6} M of indoleacetic acid in embryo and endosperm extracts of dormant seeds of European spindle-tree (*Euonymus europaeus* L.), Norway maple (*Acer platanoides* L.), Tartarian maple (*Acer tataricum* L.) and European ash (*Fraxinus excelsior* L.). During low temperature treatment used to afterripen these seeds, a sharp drop in indoleacetic acid content was recorded. This occurred in most cases at the very end of the low temperature afterripening period. The suggestion was made that indoleacetic acid may be effective in depressing embryo growth.

Wareing (1966) has not been entirely in favor of the idea that low temperature treatment overcomes dormancy of many seeds by aiding in the breakdown and disappearance of inhibitory substances in the seed. He stated, in fact, that there is seldom an appreciable decrease in inhibitor levels during low temperature treatments.

B. Growth promotors

In addition to temperature, seed germination can be influenced by other stimuli such as light or chemical substances. Some of the chemical substances may occur naturally in seed.

Though not directly related to the studies reported in this dissertation, light effects in relation to seed dormancy cannot be overlooked. Some seeds, for example, lettuce (*Lactuca sativa* L.), germinate readily after being subjected in an imbibed state to light of a wavelength in the region of 670 mμ (Evenari, 1965). On the other hand, the promotional effect is reversed by subjecting this seed to light of a wavelength of 730 mμ. Seed of white birch (*Betula pubescens* Ehrh.) behaves in a manner similar to that of lettuce when exposed to light (Black and Wareing, 1954). It has been established that the light sensitive pigment phytochrome, a bluish-green protein, is involved in controlling the germination of such seed (Borthwick and Hendricks, 1960).

Interactions and substitutions by chemical substances for light and temperature after-ripening of seed are known. In fact, low temperature pretreatment of lettuce seed at 2° C for three days can substitute for red light induction, bringing about germination of lettuce in the dark at 26° C (Ikuma and Thimann, 1964). Nitrates, especially

potassium nitrate, and other inorganic substances are known to replace the light requirement of some light sensitive seed for germination (Toole *et al.*, 1956) as in the case of peppergrass (*Lepidium virginicum* L.). Moreover, nitrate containing compounds have been used to enhance germination of non-photosensitive seeds of herbaceous plants (Mayer and Poljakoff-Mayber, 1963), such as Speedwell (*Veronica longifolia* L.) and sorghum (*Sorghum* sp.).

In addition to nitrates, a sulfur containing compound thiourea, has been found effective in promoting germination of lettuce seed in the dark (Evenari, 1965). Johnson, according to Mayer and Poljakoff-Mayber (1963) has noted that thiourea can substitute for low temperature after-ripening of non-photosensitive seeds such as oak (*Quercus* sp.), larch (*Larix* sp.) and spruce (*Picea* sp.).

Gibberellic acid has been shown to promote germination of dormant seeds of many woody plants. Jarvis *et al.* (1968a) have found that germination of the non-after-ripened seeds of hazel-nut can be promoted by treatment with gibberellic acid. This is contrary to the experience of Corns (1960) who suggested "an appropriate state of 'ripeness' is required before gibberellin can hasten or improve the percentage germination by overcoming inhibiting substances or processes." Burns (1967) found that the seed of such woody plants as oak, maple, ash and sweet gum (*Liquidambar styraciflua* L.) responded quickly and favorably to this substance without after-ripening, however, there was no indication of the physiological maturity of these seeds.

Curtis and Cantlon (1968) made an interesting observation on the effect of gibberellic acid on the germination of the arboreal

parasite, cow wheat (*Melampyrum lineare* Desr.). Seed of this plant germinates naturally only in response to highly complicated environmental conditions and yet treatment with gibberellic acid was able to completely substitute for these.

Not only has exogenously applied gibberellin promoted germination of dormant seed, it has also been known to promote growth of dormant epicotyls. Barton and Chandler (1957) showed that gibberellic acid, when applied to tree peony seedlings at rates of 10 μg and 100 μg of gibberellic acid per seedling, overcame epicotyl dormancy in 80% of the seedlings in three weeks. Gibberellic acid by-passed the seedlings' requirement for several months of low temperature after-ripening to break the dormancy of the epicotyl.

C. Mechanisms underlying dormancy and germination

Amen (1968) has stated that a balance of natural growth inhibitors and promotors is responsible for the induction, maintenance and cessation of dormancy in seeds. He has proposed a model involving an inhibitor-promotor complex as the common regulatory mechanism of the dormancy phases. When the condition favors an inhibitor, dormancy is induced. It is then maintained until the condition is shifted to favor a promotor. At this time dormancy ceases and germination is triggered. This shift within the complex to favor a promotor may be the result of either a degradation of the inhibitor or a synthesis of the promotor.

If such an inhibitor-promotor complex exists, how do the growth regulatory substances exert their influence to effect dormancy or germination? Little is known about the inhibitory aspect of Amen's

proposed complex, however, plausible explanations have been advanced by several workers to account for the mode of action of the promotor. Van Overbeek (1966) postulated that gibberellins, cytokinins and auxins were involved in promoting seed germination by stimulating synthesis of nucleic acid and hydrolytic enzymes. More recent evidence, advanced by Jarvis *et al.* (1968b), favors gene repression as being the means of imposing dormancy with derepression accompanying the breaking of dormancy. Whether gene repression is brought about by an inhibitor or by the lack of a growth promotor is uncertain. It has been suggested, however, that gibberellic acid may be one of the growth regulators involved in derepressing genetic information (Jarvis *et al.*, 1968b). In fact, Jarvis has shown that treating dormant seed of the hazel-nut with gibberellic acid resulted in a rapid increase of nucleic acid synthesis. This synthesis appeared to be centered in the embryonic axis. The rate of nucleic acid synthesis is controlled by the amount of available DNA for transcription as well as RNA polymerase activity but it has not yet been shown that gibberellic acid affects the transcriptional process directly (Jarvis *et al.*, 1968a).

Excised Embryo Behavior on Substrates

Flemion (1937) has suggested that no matter how deep the dormancy of a seed, its embryo would show growth on moistened filter paper. On the other hand, Villiers and Wareing (1965) have noted that dormancy of excised embryos may continue due to a lack of growth promoting substances or to the presence of growth inhibitors or to a combination of both.

In the culturing of excised embryos, the source of nitrogen in the growing medium has proven to have had greater significance than the source of carbohydrate. Stokes (1953b), using combinations of nitrogen and carbohydrate from varied sources for culturing embryos of cow parsnip, found that growth was most successful in those combinations where potassium nitrate was the nitrogen source. Although the results were not as striking, successful growth was also obtained when the amino acids arginine and glycine were used as the nitrogen source. Curtis and Cantlon (1968) also obtained excellent growth in culture of excised embryos of cow wheat with potassium nitrate as the nitrogen source. The amino acids glycine, arginine and glutamic acid, used individually, produced growth of embryos but only about half of that produced when potassium nitrate was used.

Van Overbeek *et al.* (1942) showed that jimsonweed (*Datura* sp.) embryos could be grown in a strictly inorganic medium without sugar. Stearns and Olson (1958) found that embryos from dormant seeds of Eastern hemlock (*Tsuga canadensis* (L.) Carr.) showed rapid and normal development on a complete tissue culture medium. Morholt *et al.* (1966) suggest that White's culture medium containing the macronutrients, micronutrients, vitamins and the amino acid glycine should be satisfactory for the growth of most excised embryos.

Growth regulating substances have also been used with excised embryos. Daletskaya (1964) found that indoleacetic acid, at concentrations of 1×10^{-4} M to 1×10^{-6} M, inhibited growth of European spindle tree embryos but more dilute solutions had no effect. The inhibitory concentrations were found by Daletskaya to be present in the dormant seed.

Nekrasova, according to Stokes (1965), obtained good growth and development of dormant peach (*Prunus persica* Sieb. & Zucc.), apricot (*Prunus armeniaca* L.) and sweet cherry (*Prunus avium* L.) embryos when these were treated with 100 ppm gibberellic acid for 48 hours. Treatment of whole seeds, however, was ineffective in breaking dormancy.

Raghavan and Torrey (1964) found that addition of either gibberellic acid or indoleacetic acid to a complete culture medium effectively promoted root and hypocotyl growth of both mature and immature Shepherd's purse (*Capsella bursa-pastoris* Medic.) embryos. Gibberellic acid was, however, more effective than auxin in promoting growth.

MATERIALS AND METHODS

I. Seed Source

Seed of the American highbush cranberry (*Viburnum trilobum* Marsh.) used in these studies was obtained from fruit harvested in 1967 and 1968 from plants growing on the University of Alberta campus. Seeds were extracted from the pulp, rinsed, air dried and stored in the dark at 2 - 3° C with the endocarp intact. Viability tests were conducted on the seeds after six months in storage.

II. Nature of the Seed Endosperm

Studies preliminary to the investigation of after-ripening in highbush cranberry seed were made on endosperm material from the 1967 harvest.

A. Total lipid

Extraction, purification and determination of total lipid is outlined in detail in Section IV (B and C). Extractions were made in triplicate on 0.6 g samples of ground, oven-dried endosperm.

B. Total nitrogen

Total nitrogen was determined on 15 mg samples of fat-free residue using the micro-Kjeldahl digestion method outlined by Koch and McMeekin (1924), with the exception that 3 ml of concentrated sulfuric acid, 0.5 g of potassium sulphate and 40 mg of mercuric oxide were added to each Kjeldahl flask. Nitrogen determination of digest aliquots was carried out by Nesslerization (Paech and Tracey, 1965). Spectrophotometric

readings were made at 390 mμ. A standard curve for nitrogen was prepared after nesslerization of known amounts of ammonium sulphate (Ward and Johnston, 1962).

C. Free amino acids

Methods of extraction of amino acids were adapted from those of Sane (1968) and of Cossins and Beevers (1963).

One hundred seeds of the 1968 stock were decorticated and the embryonic tip of the seed discarded. Endosperm material was dried in a vacuum oven at 60° C for 16 hours and then ground on a Wiley mill to pass a 20 mesh sieve. A 0.5 g sample of the oven dry endosperm was extracted with 10 ml of 80% ethanol by grinding in a mortar for five minutes. The ground material and rinsings were transferred to a centrifuge tube, brought to 13 ml with 80% ethanol and centrifuged for 15 minutes at 16,000 x g. The supernatant was decanted and the residue resuspended in 13 ml of 80% ethanol for five minutes. The preparation was again centrifuged and the procedure repeated two additional times. The supernatants from the four centrifugations were combined and evaporated to dryness under vacuum at 40° C.

Following evaporation, the residue was taken up in 20 ml of distilled, demineralized water and the lipids were removed by extraction in a separatory funnel with 15 ml of anhydrous ether. Ether extraction was repeated seven times.

The organic acids and sugars were removed from the lipid-free extract by passing the entire 20 ml of extract through a 1 x 6 cm column of BioRad 50W-X8, 200-400 mesh cation exchange resin (Calbiochem). The

column was then washed with 40 ml of distilled water to remove the sugars and organic acids. Finally the amino acids were eluted with 40 ml of 2 N ammonium hydroxide. This fraction was evaporated under vacuum at 40° C and the residue was redissolved in 2 ml of distilled, demineralized water and immediately analyzed. To determine the quantities of free amino acids present in the endosperm of the highbush cranberry, 300 µl of the final fraction were analyzed with a Beckman-Spinco model 120 amino acid analyzer.

III. Germination Experiments

Seeds used were decorticated, by removing the endocarp, just prior to planting. Unless otherwise stated, decorticated seeds were given five rinses before planting, to remove any water soluble germination inhibitor adhering to the surface of the testa (Knowles and Zalik, 1958). Rinsing involved shaking the material in distilled water for a two minute period, following which the liquid was discarded and fresh water added.

The techniques of planting were those of Knowles and Zalik (1958). Seeds were placed in perlite in 1/4 pint 'Sealright' (Sealright Can. Ltd.) waxed paper food containers. Twenty-five seeds were planted in each container. The perlite was moistened weekly and the excess free water poured off. Tops of the containers were perforated to permit air exchange.

Two lines of investigation were followed in the germination studies to determine: (1) the effect of prolonged exposure to constant 20° C on germination and (2) the effect of alternating temperatures on the time required for maximum germination.

Two replicates of 100 seeds each, from the 1967 crop, were planted one month after harvest and placed to germinate in the dark at 20° C. Seed was examined at weekly intervals and germination counts recorded over a 54 week period.

In the second experiment, the effect of a series of nine treatments, consisting of zero to eight cycles of alternating temperature, was studied in relation to the length of time subsequently required for germination when seeds were placed in constant 20° C. Each cycle of alternating temperature consisted of one week of 20° C followed by one week of 2° C, hence, seeds receiving one cycle of alternating temperature received a total of two weeks of treatment before being placed in constant 20° C temperature. On the other hand, seeds receiving eight cycles of alternating temperature were exposed to this treatment for 16 weeks prior to being placed in the constant temperature environment.

In this experiment all work was carried out with imbibed seeds. Sample size consisted of 25 seeds each and the treatments were replicated four times. Radical emergence was observed and recorded until seed from all treatments had shown 92% germination.

IV. Experiments Involving Gibberellic Acid (GA_3)

A. Effect on germination

An investigation was undertaken to determine whether GA_3 would promote the germination of seed that had not been after-ripened. Decorticated, rinsed and unrinsed seed, therefore, was treated by soaking in GA_3 (Eastman Kodak, 80^{+}%) for 24 hours, planted and placed to germinate in the dark at 20° C constant temperature. Four treatments were used.

These consisted of four concentrations of GA_3, specifically 0, 100, 300 and 500 ppm. Samples were made up of 25 seeds each and each treatment was replicated four times.

A second experiment, to determine the effect of GA_3 on the germination of after-ripened seed, was also conducted. Seed for this experiment was selected from ungerminated material which was being maintained at 20° C constant temperature after having been after-ripened by four cycles of weekly alternating temperature (20° C and 2° C). Seeds were treated by soaking in GA_3 for 24 hours, planted and returned to germinate in the dark at 20° C constant temperature. Three treatments were used. These consisted of three concentrations of GA_3, specifically 0, 100 and 300 ppm. Samples were made up of 10 seeds each and each treatment was replicated four times.

B. Effect on excised embryos

Untreated seeds as well as seeds that had been exposed to germinative conditions at 20° C for 170 days were used as embryo sources. Seeds were surface sterilized in 1% sodium hypochlorite for 15 minutes and then rinsed six times with sterile distilled water prior to embryo excision. Standard aseptic techniques were observed in the course of excision.

Treatments consisted of two media: (1) White's medium with sugar (Difco) plus 1000 ppm GA_3 and (2) White's medium with sugar. Twelve embryos were used per treatment. Comparison was made with embryos which had been cultured in distilled water.

Single embryos were transferred to 6 x 50 mm culture tubes containing 0.6 ml of medium. The culture tubes were stoppered with 8 x 15 mm 'Dispo-plugs' (Canlab), rotated gently to move the embryos to within a few millimetres of the liquid-air interface, and then placed on their sides in a 9 cm petri plate. A smaller, 20 mm petri plate of sterile distilled water was placed in the larger petri plate to maintain humidity and prevent evaporation of the medium. The larger petri plates used were covered and kept in the dark at room temperature.

Lengths of embryos were measured both at the time of excision and after seven weeks in culture. A microscope equipped with a filiar eyepiece was used for measuring.

C. Effect on epicotyl growth

Because highbush cranberry seedlings show epicotyl dormancy, GA_3 was used in an attempt to break dormancy and induce growth.

Newly germinated seedlings of highbush cranberry with roots 20 - 25 mm long were planted, 11 to a six-inch clay pot, in a sterilized mixture of soil, granulated peat moss and sand (2:1:2). Seedlings were planted so that only the cotyledons, with remnants of the endosperm and testa adhering, plus a few millimetres of the hypocotyl showed above the soil.

Gibberellic acid was applied to the base of the hypocotyl of each seedling. A 1 g/L solution of GA_3 was prepared. Treatments consisted of two rates of application. In the first, the solution was applied, 5 µl at a time, to give 10 µg GA_3 per seedling. In the second, the GA_3 was applied in the same way at the rate of 100 µg per seedling.

Approximately 15 or 20 minutes was required between applications. To prevent excessive drying of exposed seedlings during the application period, a large petri plate was inverted over the pots.

Following application of the GA_3, that portion of each seedling above the surface was covered to a depth of 0.6 cm with moist soil. The treated materials, replicated twice, along with two untreated checks were placed in the greenhouse and maintained at 21 - 22° C under normal October daylight conditions. Observations were made at regular intervals and, as soon as seedlings re-emerged from the soil, the adhering testa and endosperm were cut away to expose the cotyledons.

V. Effect of Germinative Conditions on Lipid Changes

Because lipids are well known structural components of new tissue and must be utilized during germination, any appreciable change in the components of the lipid fraction during after-ripening or on exposure to germinative temperatures could signify the start of germination. In this study the polar and non-polar lipids of the seed of the highbush cranberry were observed periodically during exposure to both after-ripening and germinative temperatures in an attempt to pinpoint the time at which germination begins.

A. Material for lipid studies

Lipid analyses were made at 30 day intervals for a period of 120 days on non-after-ripened seed, on after-ripened seed and on seed undergoing after-ripening treatment. Only in the case of seeds at time zero and in the case of after-ripening seeds at 30 days, were extracts made from seeds not in the 20° C germinator.

For each analysis, packages were selected at random and samples of 50 - 60 seeds each were collected in duplicate. On sampling dates where germination was evident, the proportion of germinated seeds per sample was kept uniform between replicates. The material was dried in a vacuum oven at 60° C to constant weight.

B. Extraction

The dried material was ground in a Wiley mill to pass a 20 mesh sieve. A 0.5 - 0.6 g sample was extracted for total lipid in each case. The method used was adapted from Redshaw (1968).

To each sample in a 5 ml boiling flask was added 4.5 ml of a 2:1 chloroform-methanol mixture. The mixture was shaken and allowed to stand overnight at room temperature. After a three minute reflux, it was filtered, with the aid of a vacuum, through a 10 - 15 μ sintered glass funnel.

The filtrate was evaporated to dryness with a stream of nitrogen. Heat was supplied from a small hot plate and, to prevent overheating, the flask was held in a tray containing a 2.5 cm layer of glass beads. The residue was redissolved in ethanol-free chloroform made up according to the method of Redshaw (1968) and then filtered through a layer of Hyflo Super-cel (Fisher Sci.) on a 10 - 15 μ sintered glass funnel to remove the bulk of protein and starch impurities. The solvent was evaporated with a stream of nitrogen. To precipitate any further protein, the residue from this filtration was treated with hot methanol which was then removed by evaporation with nitrogen. Treatment with hot methanol was carried out three times. The residue was redissolved in ethanol-free

chloroform and the mixture drawn through a layer of anhydrous sodium sulphate to remove solid particles and traces of water. The ethanol-free chloroform was evaporated off in a nitrogen stream until constant weight was obtained. This was recorded as the weight of total lipids.

The total lipid extracts were taken up in 2 ml of chloroform and transferred to screw capped vials which were stored at -20° until fractionation.

C. Fractionation

The apparatus for fractionating lipid material was made up of a 2.3 x 25 cm chromatography tube to the base of which was fitted a 25 - 50 μ sintered glass funnel and Teflon stopcock. A 200 ml reservoir was fitted to the top of the tube. Since separations frequently occur in adsorption columns when temperatures become too high, a water jacket was fabricated around the tube so that the temperature of the adsorbant might be kept at 18° C.

Chromatography grade silicic acid (100 mesh, Mallinckrodt) and Hyflo Super-cel were prepared according to the method of Redshaw and Zalik (1968).

A mixture of 20 g of silicic acid and 10 g of Hyflo Super-cel was slurried in 80 ml of ethanol-free chloroform and added to the chromatography tube. The mixture was allowed to settle overnight. The surface of the ethanol-free chloroform was allowed to fall to the level of the mixture following which the column was ready for use.

Total lipid, in the amount of 150 - 250 mg in 5 ml of ethanol-free chloroform, was applied to the column. The non-polar lipids were eluted from the column bed with 150 ml of ethanol-free chloroform and the polar lipids with 150 ml of a 1:2 chloroform-methanol mixture. Compressed nitrogen was used to control the rate of flow of the eluant through the column to 2 ml per minute.

Each of the two fractions was concentrated almost to dryness under vacuum at 35° C. The non-polar lipid fraction was transferred to a 5 ml boiling flask and together with rinsings was evaporated to dryness in a nitrogen stream. After weighing, the fraction was taken up in ethanol-free chloroform using 0.5 ml chloroform to 100 mg of non-polar lipid. It was stored under nitrogen at -20° C until required.

The polar lipid fraction was handled in similar fashion but because the eluant dissolves small amounts of silicic acid from the column, the fraction was further purified following the method of Redshaw and Zalik (1968). The polar lipids were then weighed, redissolved in ethanol-free chloroform using 0.1 ml chloroform to 1 mg of polar lipid. It was stored under nitrogen at -20° C until required.

D. Thin-layer chromatography

Glass plates, 20 x 20 cm precoated with 250 μ thickness silica gel F-254 (Merck), were used for chromatography. Vertical lines were drawn with a spatula on the plates to provide nine 'lanes' on each (Skipski *et al.*, 1965). The plates were activated by heating at 105° C for 30 minutes. The technique used was one dimensional ascending chromatography and the solvent front was allowed to run for a distance

of 15 cm. The chromatographic chamber (Desaga) was lined with Whatman No. 2 filter paper and wetted with the developing solvent 10 minutes before insertion of the thin-layer plate.

Samples were applied to the thin-layer plate 2 cm from the bottom edge with a microsyringe. A total of 45 µl of polar lipid solution was applied as five overlapping spots, to each 'lane' of an activated plate, so that the applied material took the shape of a 3 x 6 mm bar. Similarly, 10 µl of non-polar lipid was applied to each 'lane' of another plate. Following application of lipids, chromatograms were run using chloroform as the solvent in the case of non-polar lipids and a 75:25:4 chloroform-methanol-ammonium hydroxide mixture (Horrocks, 1963) as the solvent in the case of the polar lipids. The chromatograms were sprayed with 50% sulfuric acid and the spots made visible by heating the plates at 80° for 15 minutes (Nichols, 1964).

VI. Plant Growth Regulating Substances in Seed Coverings

The presence of plant growth regulators in the seed coverings of the highbush cranberry has been recognized, however, no attempt has been made to determine the number of such substances and their effects on germination.

A. Bioassay of seed testa and endocarp extracts

The effect of water extracts on bioassay material was studied according to the method of Scifres and McCarty (1969). Extracts of the testa were prepared by intermittent shaking for five hours, of samples of 200 decorticated highbush cranberry seeds in 50 ml of distilled water. Endocarp material was extracted in a similar manner only in this case it

was the ground endocarp of 200 seeds which was added to 50 ml of the extractant.

Four concentrations (0, 1, 25, 100%) of each extract in distilled water, were used in the experiment. Bioassay was performed by applying 6 ml of each concentration to seed in petri plates lined with Whatman No. 2 filter paper. Each petri plate contained 25 seeds of either cucumber (*Cucumis sativa* L. var. Straight 8) or wheat (*Triticum vulgare* L. var. Manitou). All seeds used in the bioassay had been surface sterilized in a 1% sodium hypochlorite solution for 15 minutes and then rinsed five times in sterile distilled water. Two samples of 25 seeds each of both seed types were used with each concentration. Samples were placed in the dark at 20° C. Germination and root and shoot measurements were made after five days.

B. Stability of the growth regulating material

Extracts of both testa and endocarp of highbush cranberry seed were subjected to both boiling and freezing temperatures prior to being used in a bioassay. The extracts were prepared in the manner already cited.

Three concentrations, 1, 25, 100% of each extract were prepared. Each concentration was divided into three aliquots, one of which was boiled for 15 minutes, the second quick frozen for one hour and the third was untreated.

Extracts were applied to wheat and cucumber seed for bioassay as previously described. Germination counts and seedling measurements were recorded after five days exposure to 20° C in the dark.

C. Fractionation of testa extract by gel-filtration

To determine the number of separate components in the crude testa extract that show growth regulatory activity on bioassay material, gel-filtration of the testa extract of highbush cranberry was carried out.

1. Preparation of the gel-filtration column

A 1.5 x 90 cm glass column for gel-filtration (Pharmacia) was filled with Sephadex G-15 (Pharmacia Fine Chemicals, Uppsala) which had been allowed to swell in 0.1 M phosphate buffer for 24 hours. The performance of the column and the void volume were checked by passing through a solution of Blue Dextran 2000 (Pharmacia). Fractions were collected at room temperature using an LKB model 7000 fraction collector (LKB Produkter, Stockholm-Bromma).

2. Preparation and fractionation of the extract

A water extract was prepared in the manner already cited. A 50 ml volume of extract was evaporated to dryness under vacuum at 40° C. The residue was taken up in 2 ml of distilled water and drawn through a 0.4 μ microporous filter (Millipore Corp.).

The filtered, concentrated testa extract was then loaded onto the Sephadex column and eluted with 0.1 M phosphate buffer. The flow rate was adjusted to 5.5 ml per hour and the eluate collected in 3.1 ml fractions. Optical activity of the fractions collected was determined in U.V. light at 253 mμ and 280 mμ using a Beckman DK-1 spectrophotometer.

3. Bioassay

Six millilitre samples of the eluate, representing the fractions contributing to each peak of activity indicated by the U.V. spectrophotometer, were used for bioassay. Twenty-five seeds of wheat were used to test the activity of each sample. A buffer control was bioassayed simultaneously. Seedling response, following seven days in the dark at 20° C was recorded as percentage germination and as extension growth of root and shoot.

RESULTS AND DISCUSSION

I. Nature of the Seed Endosperm

The endosperm of the American highbush cranberry was found to contain 36.4% total lipid per gram of dry weight. This is considerably more than the 25.17% total oil reported by Knowles (1957) or the 11.5% reported by Schuette and Korth (1940).

The increase in total lipid reported here can be attributed largely to improved methods of extraction. A chloroform-methanol mixture was used as the extractant, whereas, petroleum-ether was used in both cases by the workers cited. No explanation for the higher yield from Alberta grown cranberry seed has been offered, however, such differences are to be expected since seed in both cases originated from two different climatic regions.

Total nitrogen in the endosperm was 4.17% of the fat-free residue. This is slightly greater than the 3.89% total nitrogen reported by Knowles (1957) for the 1956 seed and is small enough to be attributed to differences in growing season.

Sixteen free amino acids were detected in the endosperm extract. The ones that are present in greatest amount are alanine, serine, glutamic acid, arginine and proline (Table 1).

Splittstoesser (1969 a,b) has suggested that high amounts of arginine may be required for protein synthesis during germination of some seed. Splittstoesser also noted that, in most seed of dicotyledonous plants, including woody material, arginine constitutes 16 - 18% of the

TABLE 1

Free amino acids of the endosperm of *V. trilobum* seed held in storage†

Amino acid	µmoles/g endosperm (oven dry wt. basis)	% of total free amino acids
Alanine	103.04	25.53
Arginine	50.25	12.45
Aspartic acid	35.50	8.80
Glutamic acid	67.77	16.79
Glycine	3.84	0.95
Histidine	4.76	1.18
Isoleucine	2.68	0.66
Leucine	2.24	0.55
Lysine	2.78	0.69
Phenylalanine	3.41	0.84
Proline	38.62	9.57
Serine[a]	80.82	20.02
Tryptophan	1.15	0.36
Tyrosine	1.45	1.32
Valine	5.32	0.28
Total	403.62	99.99

† - Report of one determination only.

[a] - Serine and threonine peaks are not clearly separated and are reported as serine.

total amino acids. In conifers, Durzan and Chalupa (1968) consider arginine to be an important source of nitrogen specifically for embryo growth and have reported it to be the most abundant free amino acid in the endosperm of coniferous seed.

On the other hand, Stokes (1953b) found glycine and asparagine to be the two amino acids necessary for growth of cow parsnip embryos. Hence, one cannot anticipate that something which is necessary for one kind of embryo is of equal significance to another. Unfortunately, the specific amino acids which will promote the growth of highbush cranberry are not known.

II. Germination Experiments

Knowles and Zalik (1958) showed that seeds of highbush cranberry would germinate slowly at 20° C constant temperature and further, that only 63% would germinate within a 156 day period. They also showed, when such seeds were after-ripened by being subjected to 110 days of weekly alternating temperature (20° C and 2° C) before being placed into the constant 20° C temperature for germination, that 97% of the seeds produced seedlings in 46 days.

Knowles and Zalik made no attempt to determine the precise length of the after-ripening requirement neither did they carry their control treatment, constant 20° C, for any longer than 156 days. Because of this, the period of observation in this study was extended until such time as all the seed had germinated. It required 380 days to germinate 97% of the seed when kept at germinative conditions of 20° C constant temperature.

Figure 1 illustrates the heterogeneous nature of highbush cranberry seed. It shows that, initially, only a little more than half the seed is capable of responding to optimal germination conditions. The balance of the seed, obviously, has some benefit to be received from the after-ripening treatment. Examination of the curve for treated seed shows that after-ripening completely eliminates that 120 day period exhibited by untreated seed during which there is little, if any, germination response. This lag exhibited by the non-after-ripened seed which is followed by a second surge of germination would appear to indicate one thing, that the threshold for germination in that portion of the seed lot in which visible activity is delayed was not reached at 20° C for a further 120 days. It is difficult to imagine that such a threshold might be reached over this period as the result of the accumulation of available food or of a germination promotor because it is difficult to conceive any reason why an accumulation of such materials should occur without any alteration in treatment. Rather, it seems more likely that some germination inhibiting substance might be degraded with time so that the processes leading to germination may be allowed to proceed.

If complete germination is dependent on degradation of a germination inhibitor or, for that matter, on the ability of a growth regulator to exhibit growth inhibiting properties under one particular set of conditions and growth promoting properties under another, then it is also difficult to conceive that the fluctuating conditions of the after-ripening treatment would work any better in the degradation of a growth inhibitor or in the alteration of a growth regulator. The same,

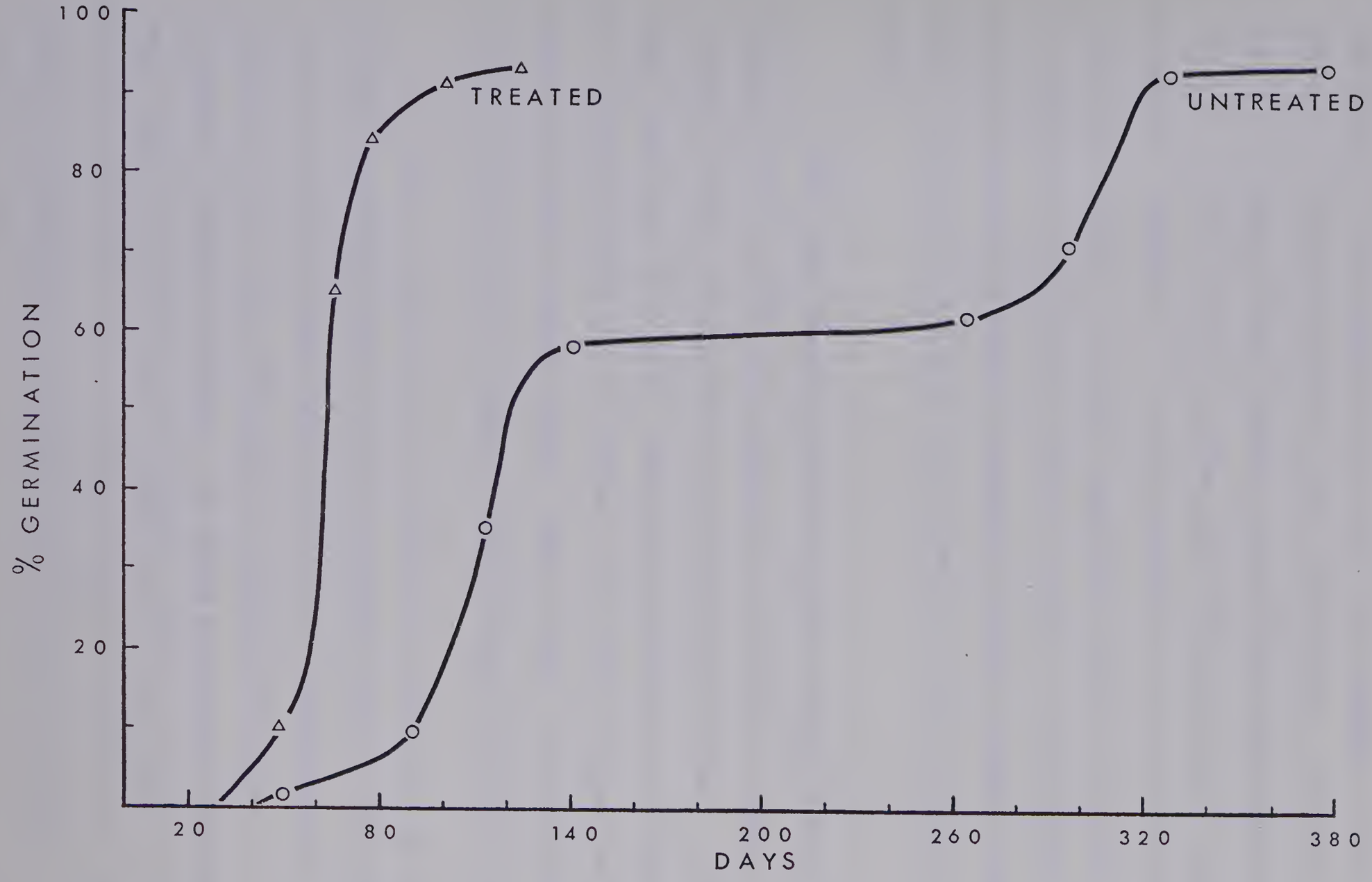

Figure 1. The effect of after-ripening on the germination of seed of *V. trilobum* at 20° C constant temperature. Treated - given 14 weeks of weekly alternating temperature (20° C and 2° C) prior to exposure to germinative conditions.

however, cannot be said for the possibility that the fluctuating temperatures of after-ripening play a part in the breakdown of food reserves and the accumulation of soluble food materials. Certainly, there is no reason why such treatment could not have some cumulative effect on the embryo through a pathway that alternates between catabolic and anabolic processes. It is easy to conceive of alternating temperatures acting this way — the lower temperature perhaps being optimal for catabolism, the higher for anabolic processes so that the embryo grows and the seedling eventually emerges following a pattern of growth, that, if graphically portrayed, would resemble a flight of ascending stairs.

While these two curves concur generally with the findings of Knowles and Zalik (1958), the response of the 1967 seed as compared to the 1956 seed indicates considerable variability between the two. To illustrate, Knowles and Zalik (1957) reported 52% germination in 50 days at 20° C constant temperature for 1956 seed. The 1967 crop showed only 2% germination within a similar period. After 80 days, less than 9% of the 1967 seed had germinated as compared to 59% for that of the 1956 crop.

This, of course, might be expected since seeds were not only harvested in different years but also at different times. Seed of the 1967 crop was harvested one month earlier than was the seed used by Knowles and Zalik. Such differences in response could be due to differences in degree of maturity. It should be noted that the average embryo length of the 1956 seed lot (Knowles, 1957) was greater by 0.04 mm than that of the 1967 lot. The average length of embryos of the 1956 seed was 0.72 mm whereas that noted in the 1967 seed was 0.68 mm.

Another possible reason for these germination differences could be attributed to a difference in the technique of handling. When germination counts were made in the present study, the germination media was removed to expose the seed and then replaced. Knowles and Zalik (1958), in making counts, discarded the used media each time counts were made. In Knowles and Zalik's work, this procedure may have contributed to dilution of the existing germination inhibitor which may have led to the accelerated germination response of the 1956 seed lots. The procedures used here would tend to retain diffused materials in the surrounding medium and so tend to maintain the original concentration of inhibitors.

Knowles and Zalik (1958) indicated that weekly alternations of temperature were superior to daily or biweekly temperature alternations in promoting germination of highbush cranberry. Since their study involved use of only one period of after-ripening, namely 110 days of weekly alternating temperature (20° C and 2° C), they gave no indication as to the minimum time required to fully after-ripen the seeds. Because after-ripening of the seed plays such an important part in the present study, an accurate determination of the minimum time required to achieve the after-ripened condition was essential.

The 1968 crop of highbush cranberry seed showed an average viability of 92% after six months in storage when tested with the indicator 2,3,5-triphenyl tetrazolium chloride (Fisher Sci.). For comparative purposes, this value was regarded in subsequent experiments as 'total germination'.

In Figure 1, the response of highbush cranberry seed to after-ripening is shown. Figure 2 illustrates the germination response of

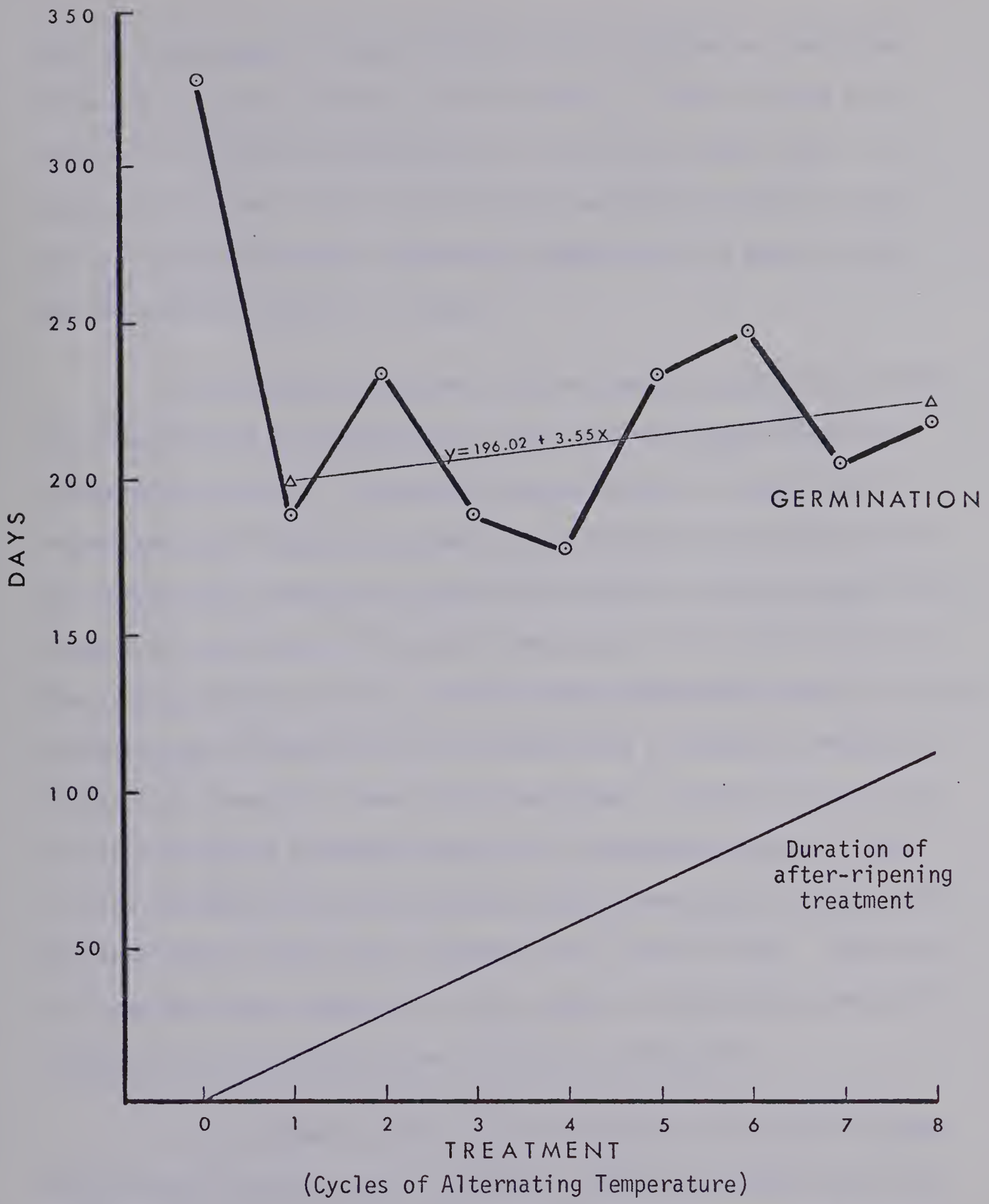

Figure 2. The effect of after-ripening on the time required for seed of *V. trilobum* to reach total germination.

each of nine samples of seed maintained under germinative conditions following different periods of after-ripening. While 328 days were required to attain total germination of non-after-ripened seed, it is interesting to note that by treating the seed with as little as one cycle (14 days) of weekly alternating temperature, the time to total germination was reduced by 129 days.

As the number of cycles of after-ripening treatment increased, the time required for germination at 20° C constant temperature was progressively reduced. The after-ripening effect, as shown by the regression line (Figure 2), appears to be somewhat cumulative; that is, the longer after-ripening is applied the shorter is the time required to reach total germination in constant temperature. This lends support to the suggestion made earlier, that fluctuating temperature may aid in the accumulation of food materials by alternations of metabolic processes. On the other hand, this shows little advantage, for the time required to after-ripen and germinate the seeds in treatments seven and eight, in fact, exceeded the time required to obtain seedlings from seeds that had been after-ripened with treatments one, three and four. These data indicate that seeds subjected to four cycles of alternating temperature require the shortest total time to complete germination.

It is noteworthy that all after-ripening treatments overcame the two seed-type phenomenon exhibited by non-after-ripened seed lots. Apparently, after-ripening of any duration is instrumental in bringing about germination in that portion of the seed lot in which dormancy persists.

III. Experiments Involving Gibberellic Acid (GA_3)

A. Effect on germination

There is a great deal of evidence (Mayer and Poljakoff-Mayber, 1963) to the effect that seeds showing poor germination when placed in germinative conditions will respond favorably to treatment with GA_3.

Figure 3 shows the response of highbush cranberry seed to GA_3, 191 days after being placed in the germinator. Analysis of variance of these data (Table 2) indicates that neither the type of seed nor the concentration of GA_3 used in the soaking process has any statistically significant effect on germination. The interaction between seed type and treatment however was statistically significant at the 5% level. An explanation for this does not present itself, however, it should be noted that germination was not promoted when rinsed seed was treated with GA_3 at any concentration. There was a slight increase in germination when unrinsed material was treated with 100 ppm GA_3 but at 300 ppm there was a noticeable decrease, an effect that was consistent at the 500 ppm level of treatment.

TABLE 2

Analysis of variance of mean squares for the effect of GA_3 treatment on the germination of rinsed and unrinsed seed of *V. trilobum*

Source of Variation	d.f.	Mean Square	F
Treatment (T)	3	17.08	3.908
Seed type (S)	1	2.25	0.514
T x S	3	31.75	7.265*
Error x Rep.	24	4.37	

* Significant at the 5% level.

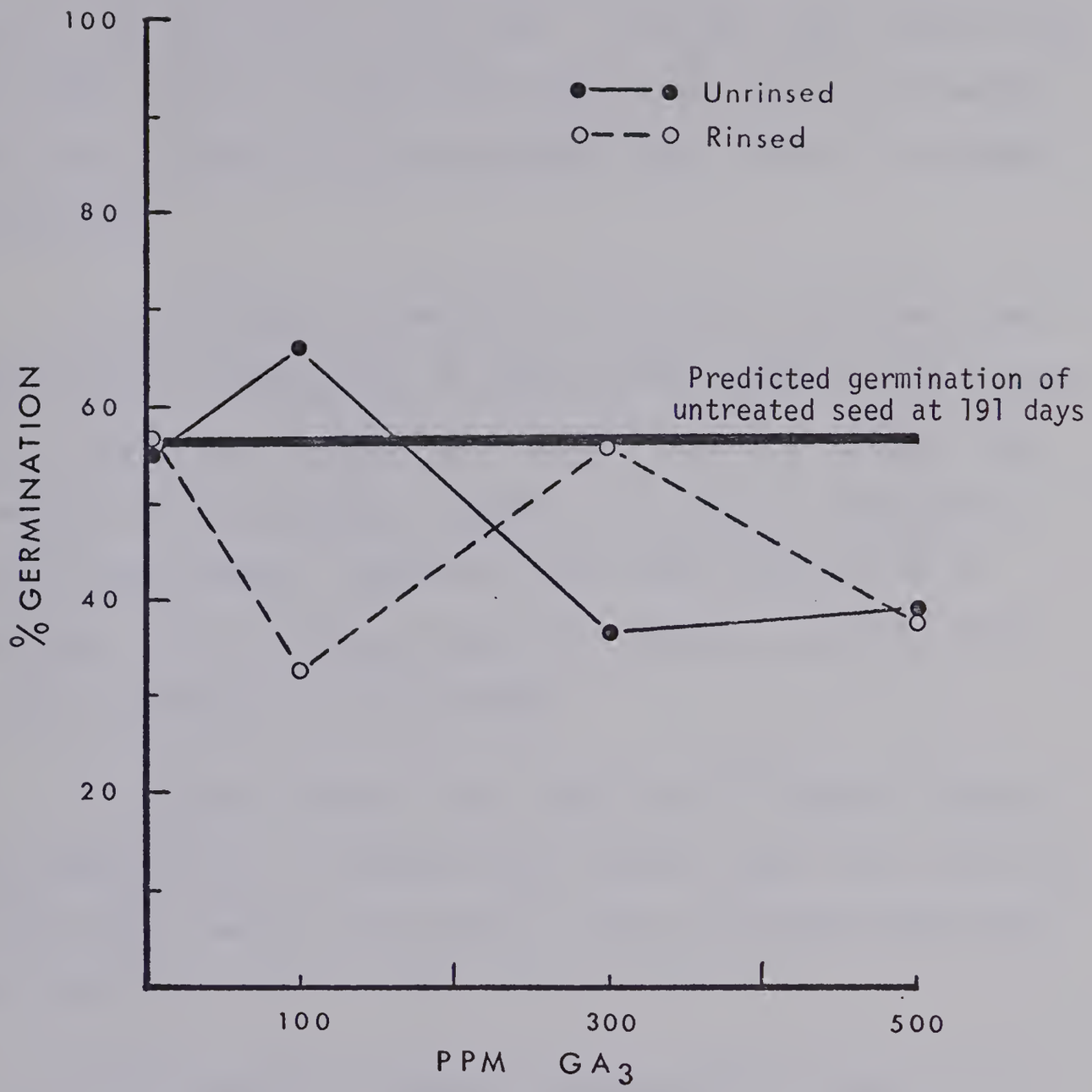

Figure 3. Germination response of non-after-ripened, rinsed and unrinsed *V. trilobum* seed to GA_3 following 191 days at 20° C. Mean germination from four replicates of 25 seeds each.

The differential response of the two types to GA_3 strongly suggests that the naturally occurring growth regulators have some part to play in the germination of this seed. It has been shown (Knowles and Zalik, 1958) that they can effectively control the rate of germination; however, this is the first evidence noted of their effect on the amount of germination.

It is not to be construed from the results of this experiment (Figure 3) that the interaction of natural growth regulators and 100 ppm of GA_3 is contributory to total germination. It merely indicates that the naturally occurring growth regulators are capable of interacting with the growth promotor, gibberellic acid, which reaffirms, in an indirect way, the observations made earlier (Knowles and Zalik, 1958) that these substances can affect growth.

To determine whether after-ripened seed of highbush cranberry would respond to GA_3, an experiment was conducted using rinsed seed that had been after-ripened by four cycles of weekly alternating temperature (20° C and 2° C).

Four weeks after treatment, no germination was observed in either the control or the GA_3 treated material. These results do not necessarily refute the possibility that seed may require after-ripening before response to GA_3 can occur; rather, in this case it is more likely that the seed used was of questionable viability. The seeds used were those which had not yet germinated after eight months in 20° C constant temperature following their after-ripening treatment.

B. Effect on excised embryos

Excised embryos of highbush cranberry were placed in nutrient media to ascertain whether growth would occur. Seed, fresh from dry storage, and seed that had been in the germinator for 170 days at 20° C constant temperature were used as embryo sources.

The embryos from stored seed averaged 0.66 - 0.69 mm in length while the embryos from germinator seed were more advanced, measuring 1.20 - 1.27 mm. The two kinds of embryos were transferred to culture media to determine:

(1) whether growth of non-after-ripened embryos could be promoted on medium, and

(2) whether GA_3 had any effect on the embryos.

No difficulty with contamination was experienced and the experiment was terminated after seven weeks, when no growth of highbush cranberry embryos was observed in either White's medium, White's medium with GA_3 or in the distilled water control treatment.

Although Morholt *et al.* (1966) has stated that White's medium is satisfactory for embryo growth, it may lack certain ingredients for the growth of highbush cranberry embryos. Villiers and Wareing (1965) have stated that continued dormancy of excised embryos may be due to a lack of a growth promoting substance. On the other hand, dormancy of the embryo might be due to an inactive enzyme system(s) for the utilization of nutrients in the medium.

The fact that GA_3 is unable to stimulate excised embryos is not entirely unexpected as GA_3 had no stimulatory effect when whole seeds

were used. On this aspect Cleland (1969) has written, "the fact that a process fails to respond to added gibberellin cannot be used as evidence that the process does not require gibberellin, since it may be a different gibberellin which regulates this process."

C. Effect on epicotyl growth

The U.S. Forest Service (1948) reported that to overcome epicotyl dormancy of highbush cranberry seedlings following root initiation, exposure to 5° C for several months was necessary. An experiment was undertaken with seedlings of highbush cranberry to determine whether application of GA_3 could be substituted for the low temperature afterripening said to be required.

The results in Table 3 show that both 10 µg and 100 µg per seedling of GA_3 induced epicotyls of this seed to elongate in the greenhouse without low temperature treatment. Figure 4 illustrates epicotyl production of seedlings, three weeks after treatment with GA_3.

TABLE 3

Effect of GA_3 treatment on growth of the dormant epicotyl of *V. trilobum* seedlings, 44 days after treatment and planting

Treatment	Growth Response
µg GA_3/seedling	No. of epicotyls/22 seedlings
0	0
10	16
100	12

Figure 4. Epicotyl production of *V. trilobum* seedlings, 21 days after treatment with GA_3. Left to right: no GA_3, 10 µg and 100 µg GA_3 per seedling at the time of planting in soil.

As was the case in Barton and Chandler's work (1957) with tree peony, 10 μg of GA_3 per seedling appeared to be slightly more effective than the higher rate of 100 μg in stimulating epicotyl growth.

At all concentrations, the appearance of the first true leaves was abnormal in seedlings treated with GA_3. The leaves were lobed but generally linear-lanceolate rather than the typical triangular shape. Similar observations were reported in experiments with tree peony.

Seedlings were maintained in the greenhouse beyond the 44 days of the experiment and it was noted that elongation of the epicotyl in treated plants ceased after approximately 15 mm of growth had been made. No further growth took place until the seedlings were approximately three months old. Then, secondary leaves began to develop and these appeared more typical than the first true leaves. By this time, the untreated seedlings had begun epicotyl growth and exhibited normal first and second true leaves. It appears that, in the case of epicotyl dormancy of the highbush cranberry, low temperature after-ripening of the epicotyl may not be the only prerequisite for growth.

IV. Effect of Germinative Conditions on Lipid Change

Non-after-ripened seed, after-ripened seed and seed undergoing after-ripening treatment with four cycles of weekly alternating temperature was sampled at intervals to determine any differences in utilization of lipid reserves. Since after-ripened seed germinates more readily (Figure 1), one would expect noticeable differences in the rate of utilization of lipids.

Because of the high initial lipid content in the endosperm of highbush cranberry (36.4%), it was anticipated that lipids would serve as the main substrate for germination. This, however, does not appear to be the case because no appreciable changes in lipids were noted. This is somewhat surprising since other workers (St. Angelo and Altschul, 1964) observed that other kinds of seeds with a high proportion of stored lipid (30 - 40%) metabolized most of this within a week after germination.

At 60 and 90 days, analyses of the after-ripened seed showed an increase in total lipid over that noted at 30 days. This was interpreted as an indication that seed constituents, other than lipids, were being utilized at this time since total lipid was based on the dry weight of the seed. Because of this, measurements of changes in total lipid could not be considered as a reliable indicator of lipid metabolism and the lipid constituent, therefore, was divided into polar and non-polar fractions and the changes in proportions of these two observed.

Two observations are to be noted in Table 4, first, that during exposure to germinative conditions, the proportions of the lipid fractions were relatively stable, and secondly, that after-ripening does not seem to have an effect on these proportions, at least within the period the seeds were observed. This is contrary to the findings of both Mayer and Poljakoff-Mayber (1963) and Redshaw and Zalik (1968) who noted that phospholipids (polar lipids) in seeds show marked increases at the expense of non-polar lipids during germination. In highbush cranberry seed, at the time of germination, there seems to be little or no synthesis of polar lipids from non-polar lipids. This is puzzling because growing tissues must have an increasing need for polar lipid

TABLE 4

Changes in lipid fractions of *V. trilobum* during after-ripening and germination†

Seed Type	Days after start of experiment	Non-polar lipids as % of total lipids	Polar lipids as % of total lipids
Non-after-ripened seed	0	95.39	4.60
	30	96.93	3.07
	60	96.74	3.26
	90	95.14	4.81
	120	94.90	5.10
After-ripening seed	0	95.39	4.60
	30	95.87	4.12
After-ripened seed	60	96.50	3.50
	90	95.67	4.33
	120	95.90	4.10

† 1968 seed stock. Average of two determinations for extraction, and separation.

at this time. Cell membranes, in particular, are dependent on a supply of polar lipids. This leads one to suspect that enzyme systems for the conversion of lipids are not generally activated until some later time.

Figure 5 shows the quantitative change in polar lipid of non-after-ripened and after-ripening seed as well as the change in polar lipid during germination of both non-after-ripened and after-ripened seed. Though interesting, the decline in polar lipid prior to germination in both after-ripening and non-after-ripened seed is small, and is not considered to be of significance. It is also interesting to note that, from the start of germination, there is a small increase in polar lipid.

Though no apparent quantitative change in non-polar and polar lipids had occurred during after-ripening and early germination (Table 4), this did not mean that qualitative changes within the two fractions themselves were not occurring. To observe such changes, thin-layer chromatography of the two fractions was carried out.

Thin-layer chromatography of the non-polar lipids (Figure 6) revealed little qualitative difference between seed held at constant temperature and that which had been subjected to the after-ripening treatment prior to being placed in the constant 20° C germinator. Not until 120 days after the start of the experiment was there any evidence of additional spots on the chromatogram. The numbers and positions of spots correspond between the two kinds of seed for any particular sampling date.

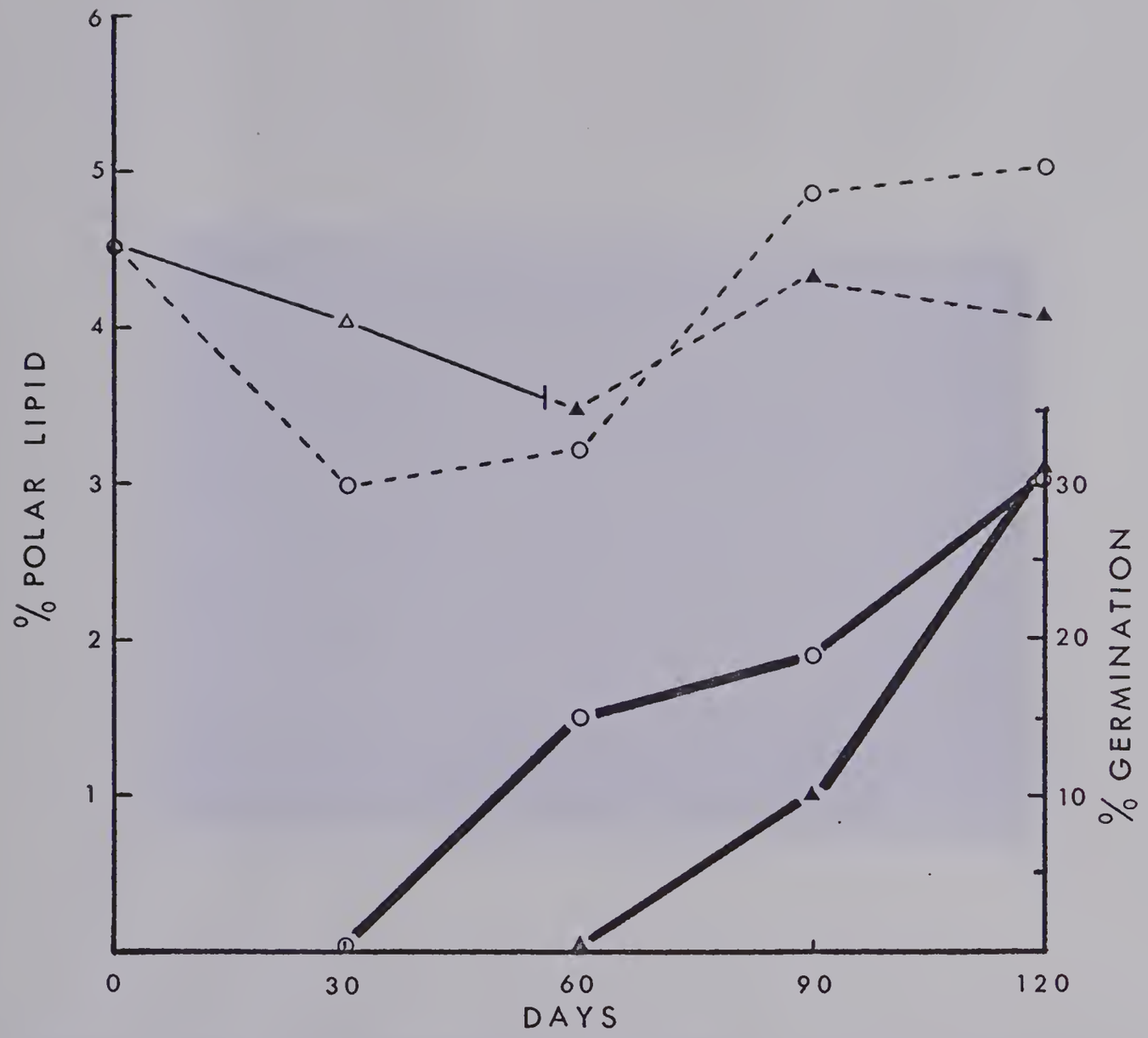

Figure 5. Observed changes in polar lipids accompanying after-ripening and germination in seed of *V. trilobum*. Non-after-ripened seed, ○; after-ripening seed, △; after-ripened seed, ▲.

Figure 6. Thin-layer chromatogram of the non-polar lipid fraction of *V. trilobum* seed sampled during after-ripening and germination. Numerical prefixes indicate days from start of experiment. A = seed subjected to after-ripening treatment; C = non-after-ripened seed.

Figure 7. Thin-layer chromatogram of the polar lipid fraction of *V. trilobum* seed sampled during after-ripening and germination. Numerical prefixes indicate days from start of experiment. A = seed subjected to after-ripening treatment; C = non-after ripened seed.

Thin-layer chromatography of the polar lipid fraction (Figure 7) from the three seed types reveals minor changes occurring as the seed is subjected to germinative conditions. The positions and the intensity of the spots change from sampling date to sampling date, indicating the occurrence or disappearance of individual polar lipids but clear differences between those of after-ripened seed and non-after-ripened seed are not apparent.

Data on total carbohydrates is not available, but it seems likely that during the early phases of germination of highbush cranberry, sugars are the most important source of energy. Knowles (1957) indicated that the endosperm was not of a starchy nature and since lipid and protein account for 62.6% of the total dry weight of the endosperm, then it would seem that sugars must constitute the major part of the remainder and must be the energy source for germination.

V. Plant Growth Regulating Substances in Seed Coverings

A. Bioassay of testa and endocarp extracts

The effect of seed covering extracts of highbush cranberry on the behavior of wheat and cucumber are shown in Table 5. Both testa and endocarp extracts exhibit growth regulating properties, however, only the endocarp extract had an effect on the germination of bioassay material. Cucumber seed germination was stimulated by the 1% concentration of endocarp extract and inhibited by the 25% and 100% concentrations. Statistically, these results were highly significant.

Removal of the endocarp from highbush cranberry seed (Knowles, 1957) shortened the time to 50% germination. Rinsing the

TABLE 5

The effect of testa and endocarp extracts of *V. trilobum* on the behavior of wheat and cucumber seed

Type of extract	Per cent media as extract	Per cent germination		Average length in mm of Roots		Average length in mm of Shoots	
		Cucumber	Wheat	Cucumber	Wheat	Cucumber	Wheat
Testa	0	78	96	37.3	67.8	7.4	32.4
	1	78	100	40.5*	73.3**	7.2	32.6
	25	78	100	24.3**	19.1**	4.3**	31.0*
	100	78	98	12.9**	13.0**	3.6**	19.4**
	$LSD_{.05}$			2.55	2.18	0.17	1.28
	$LSD_{.01}$			3.30	2.82	0.21	1.66
Endocarp	0	78	96	37.3	67.8	7.4	32.4
	1	90**	100	40.7**	69.9	7.4	34.2**
	25	74**	96	38.3	64.4	6.9	33.6*
	100	70**	96	31.5**	24.4**	4.7**	28.4**
	$LSD_{.05}$	1.18		1.45	3.74	1.39	0.97
	$LSD_{.01}$	1.52		1.86	4.81	1.78	1.26

* - Significant at the 5% level

** - Significant at the 1% level

testa further increased the rate of germination. While these studies show that the testa extract did not inhibit germination, this does not refute the work of Knowles (1957). In these studies cucumber and wheat were the test materials rather than highbush cranberry.

With regard to root and shoot growth of bioassay material in various concentrations of testa or endocarp extract, the results (Table 5) show that two different and opposite effects occur with changes in concentration. The 1% concentration of both the testa and endocarp extracts were stimulatory to root growth of bioassay material. The 100% concentration of both extracts was not stimulatory, rather, it was inhibitory to both root and shoot growth.

While a similarity in activity was noted when the effect of same concentrations of the two extracts was compared, the endocarp extract was generally less effective on both stimulation and suppression of growth than was the testa extract (Table 5). For example, considering root growth of wheat, Table 5 shows that statistically significant differences exist between three concentrations and the control when testa extract was bioassayed. Significant differences between treatment concentrations and the control occurred in one instance when endocarp extract was bioassayed only. The lesser effect of the endocarp is well illustrated by comparing Figures 8 and 9.

While it has been shown that both testa and endocarp extracts inhibit root growth, the extracts, particularly the 100% concentration, stimulate root production by wheat. The number of roots per seed in the distilled water control was 3.00 while the testa extract produced 3.82 and 3.94 roots per seed in 25% and 100% concentrations, respectively.

Figure 8. Effect of *V. trilobum* testa extracts on growth of wheat, five days after treatment. Numerical values represent concentration of extract.

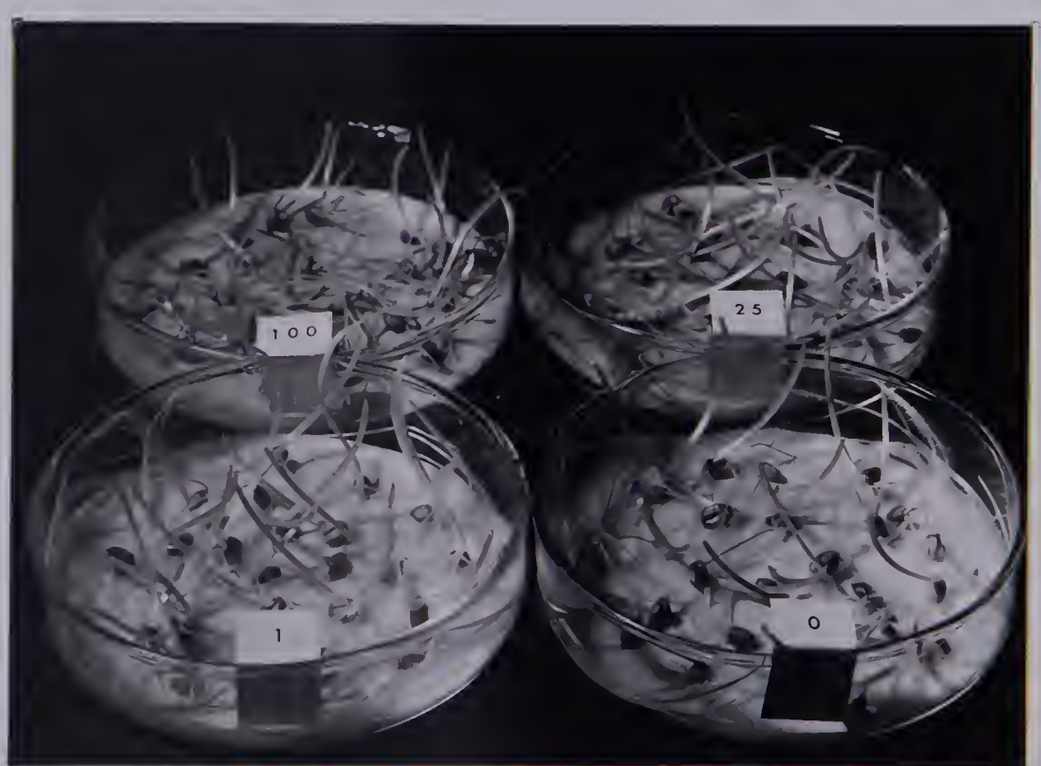

Figure 9. Effect of *V. trilobum* endocarp extracts on growth of wheat, five days after treatment. Numerical values represent concentration of extract.

When endocarp extract was used, 3.38 roots were produced per wheat seed at the 100% concentration. As in the case of growth, once again it is the testa extract that shows the greater effect. Assuming that both the endocarp and testa contain the same material, then it would appear likely that the growth substance or substances is/are more highly concentrated in the testa than in the endocarp.

As the concentration of the extracts increased, there was a noticeable increase in browning of root tips as well as the formation of 'kinks' at the root tips and swelling and browning of kinked areas. Norberg (1968) has shown wheat roots to be highly sensitive to kinetin and react to its presence by formation of abnormalities including the typical 'c-swellings' which he described as thickenings just behind the root apex. In addition, the roots curled and became claw-like. No attempt was made to identify the growth regulatory substance or substances present in the water extracts and since kinetin is not soluble in water (Strong, 1958), this response of wheat roots cannot be a specific reaction to kinetin.

From the results of these studies, some facts become apparent. Both the testa and the endocarp contain one or more growth regulating substances. Either, an inhibitor and a promotor are present, in which case the inhibitor appears to mask the action of the promotor in the higher concentrations of the extract or, a true growth regulator is present, which inhibits at high concentrations and stimulates when sufficiently dilute.

Both the testa and endocarp extracts proved to be quite stable to the effects of boiling and freezing. Analysis of variance indicated

there was no treatment effect on either extract when the effect of the two was bioassayed. Since these growth regulatory substances appear to be thermostable, then it seems quite likely that the natural growth regulators, such as auxins, are not present in the extracts because these will undergo inactivation at high temperature (Mitchell and Smale, 1963).

B. Separation of seed testa extracts by gel-filtration

In preliminary studies, testa extracts of highbush cranberry seed were passed through a column of Sephadex LH-20 and eluants monitored by U.V. spectrophotometry. Strong binding of pink pigmented substances occurred and only one peak of optical activity was observed in the eluant at 245 mμ after 600 ml of 70% ethanol had been washed through the column. Material from the activity peak was concentrated and applied to wheat seed bioassay. Germination and growth measurements were no different from the water controls.

Since bioassay indicated that no active material had been eluted, the use of Sephadex LH-20 was discontinued. It appeared likely that the molecular weights of the components of the testa extract were of a small molecular weight and were being retained in the column. Because of its lower molecular weight exclusion limit, Sephadex G-15 was chosen for subsequent filtrations. The use of this gel proved partially successful in separating the components of the testa extract.

Following thorough elution of the 1.5 x 90 cm column of Sephadex G-15, seven peaks of optical activity were determined after subjecting the fractions to U.V. spectrophotometry at two wavelengths, 253 mμ and

280 mμ. These two wavelengths were chosen on the basis that 253 mμ is the wavelength used by commercial U.V. monitoring devices for routine scanning and that absorption at 280 mμ would indicate presence of aromatic compounds. A second run of extract through the G-15 column was conducted and the results were reasonably consistent with the first.

The U.V. spectrophotometry indicated seven peaks of activity in the eluant (Figure 10), however, some doubt exists whether these were all the components of the water soluble fraction since some pink material remained bound to the column. Several low ionic strength buffers were tried in an attempt to remove the pigmented substances, but without success, hence it was not possible to achieve a complete fractionation of the water-soluble materials. Strong binding with highly cross-linked gels such as Sephadex G-15 have been encountered and recorded in the literature (Janson, 1967). Some of the more common eluting materials used in such cases of binding have been 10 to 100% ethanol, 10% formic acid, 0.02 N sodium hydroxide, 0.1 N sodium chloride, 1 M urea, 0.1 M ammonium acetate and phenol-acetic acid-water. All were tried without success. A 60% acetone solution removed some of the pink pigmented material but not completely.

From the activity noted in the bioassay (Table 6), eluant from peak No. 2 gave a four-fold increase in shoot growth and a twelve-fold increase in root growth. Eluant from peak No. 3 gave stimulation only of root growth. Eluant from peak No. 4 gave strong inhibition of shoot growth while that from peak No. 1 gave inhibition of shoot growth but not nearly as marked as that from No. 4.

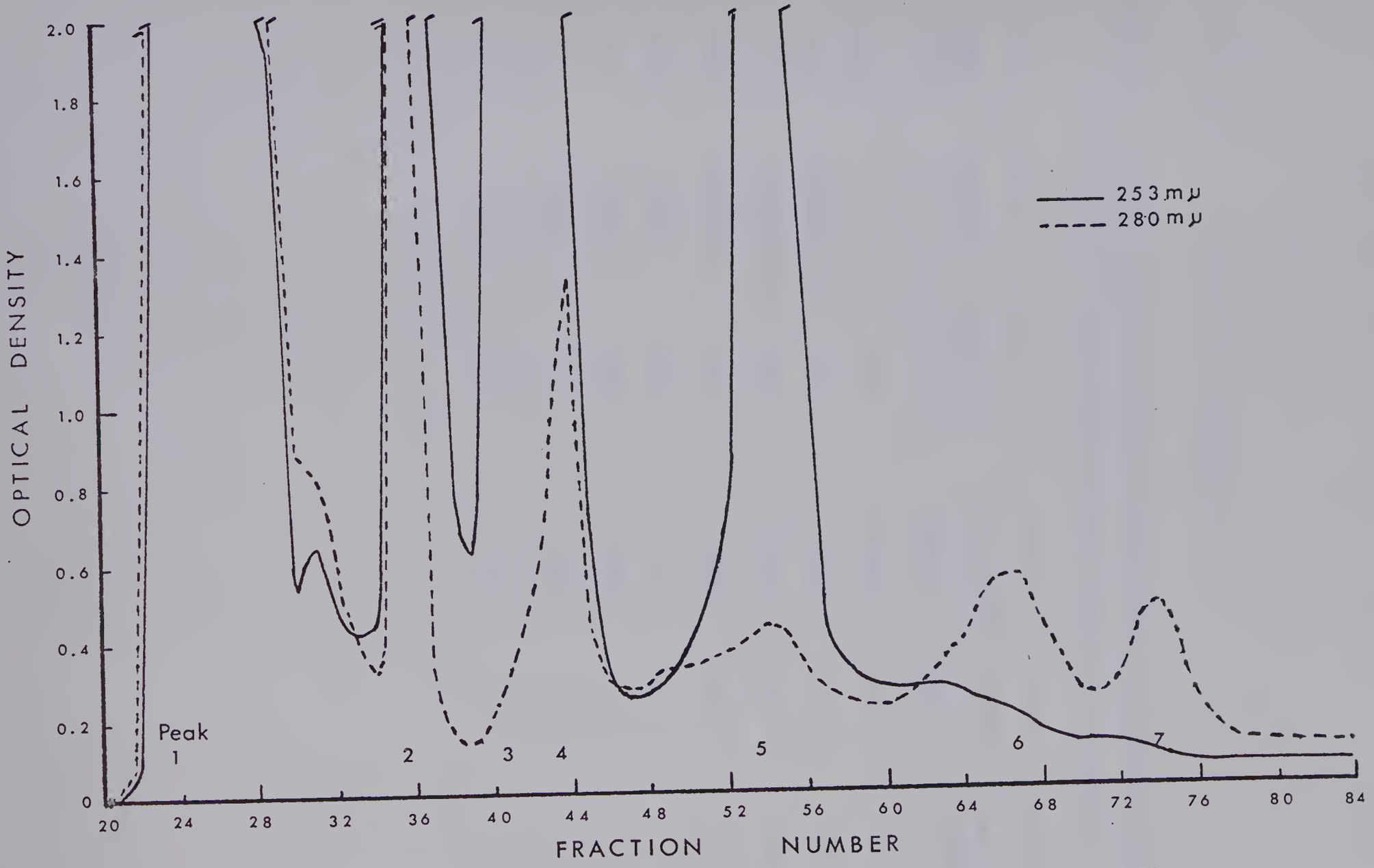

Figure 10. Absorption curves of the testa extract of *V. trilobum* monitored at 250 mμ and 280 mμ.

TABLE 6

Germination of wheat seed and growth of seedlings in eluant of 2 successive 3 ml fractions corresponding to each peak of U.V. activity

Peak number	Fraction No.	Per cent germination	Ave. length in mm of		No. of roots/seed
			Shoots	Roots	
Buffer		64	32.25	5.57	3.25
1	23-24	60	19.20	8.33	4.0
2	35-36	96	104.71	63.69	4.28
3	40-41	96	40.96	26.19	4.88
4	43-44	68	9.12	5.02	3.59
5	53-54	80	29.05	7.52	4.25
6	66-67	72	35.83	6.03	4.22
7	73-74	68	25.06	6.21	3.59

Table 6 also shows that root production was stimulated in all the fractions assayed. The eluant from the peak that gave the greatest number of roots noted per seed did not, however, coincide with that which gave the largest amount of root growth.

These studies indicate that the testa extract shown by Knowles and Zalik (1958) to inhibit root growth of wheat contains, not one, but seven or more water soluble substances which individually are capable of affecting germination and as well, stimulating or inhibiting growth of the seedling. Inhibition of root growth is not evident in these results though strong inhibition was shown by the crude extract (Table 5). It is quite possible that an interaction of all the compounds is responsible for this inhibition or that the factor responsible for marked inhibition by the crude rinse may not have been removed from the column during gel-filtration.

Molecular weight estimations of the substances eluted were not attempted due to the adsorption interaction of the gel with the extract. No further attempts at identifying the water soluble growth regulating substances were made.

SUMMARY AND CONCLUSIONS

When seed of the American highbush cranberry was maintained in germinative conditions at 20° C constant temperature, approximately 58% of the seed germinated in 140 days. This was followed by a period of 120 days during which only a few seedlings emerged. After this lag there was a second and rapid surge of germination to 97%. Total germination took place at 20° C only after 328 days in the germinator. Just why seed lots should behave in this manner was not immediately apparent. Obviously, the 42% of the seed that did not germinate in 140 days had some other requirement for germination than those of the other 58%. However, they eventually did germinate at 20° C indicating that the requirement for germination could be met by prolonged exposure to these conditions.

Although it was known that after-ripening treatment would hasten germination of the intractable portion of the seed lot, the minimum amount of after-ripening required to trigger germination in these seeds was not known. It was surprising to find that as little as 14 days of weekly alternating temperature caused a steady increase in germination, shortening the time to total germination by four and one-half months. The effect of alternating temperature was inductive; no germination occurred during after-ripening even in cases where this treatment was extended to as much as 112 days. Germination only took place after the seeds had been established in constant 20° C. Germination of highbush cranberry, then, can be conditioned by something which occurs during the after-ripening treatment. There were indications that this change was cumulative, in other words, the longer the after-ripening period, the shorter the time required in constant temperature for germination.

Attempts to determine where the effect of after-ripening was manifest, were carried out on the endosperm material during the time seed was being after-ripened. Since the endosperm was made up largely of lipid, it was expected that the effect of after-ripening would show up on this component. Since total lipid actually increased during after-ripening, it was quite obvious that some other component was being utilized. In spite of this, it was reasoned that as growth of the embryo took place, some effect might be noticed on the fractions making up total lipid, namely, the polar and non-polar lipids. It was expected that there would be noticeable changes in these fractions and that this would herald the onset of germination. Little or no change in the two lipid fractions occurred during prolonged exposure to both after-ripening and germinative temperatures. Hence, it was very difficult to conceive why this seed should have such a high lipid content and not utilize it at a time when such material is required for synthesis of new tissue. At least it can be said there must be very little lipase activity in the endosperm during after-ripening and during the early stages of germination.

Water-soluble materials found in the seed coverings of highbush cranberry are known to delay germination. Although these might have a special regulatory effect on the process, it was difficult to conceive how alternating temperature might overcome this inhibiting effect. Because of this, it seems reasonable to suggest that the effect of this type of after-ripening must be concerned with metabolic substrates, other than lipid, rather than with growth regulating substances.

Though it is difficult to determine just what part naturally occurring growth regulators might have in the after-ripening and

germination of highbush cranberry, it was decided to investigate the one growth regulator theory proposed by Knowles and Zalik (1958). Fractionation of the water-soluble extracts of the testa indicated the presence of four, rather than one, active materials, two of these showed strong growth promoting properties while two were moderately inhibitory. In view of the fact that concentrated solutions of the crude testa extract strongly inhibit growth and that very dilute concentrations have precisely the opposite effect, the evidence that more than one growth regulatory substance is involved in inhibition and promotion calls for a new look at this phenomenon. It might be that the inhibitors and promotors are differentially soluble in water, that the inhibitory substances are capable of masking those that cause stimulation and that dilution of the crude extract has its greatest effect on the inhibitors.

Gibberellic acid showed no effect on the germination of this seed and showed no stimulation of embryo growth when it was combined with the culture medium. However, when GA_3 was used on seed from which growth regulatory substances had not been removed, there was some increase in germination when the concentration of GA_3 was held to 100 ppm. Unfortunately, no lower concentrations of GA_3 were used in this experiment to test this interaction further. All that can be said is that the possibility exists that low concentrations of GA_3 may interact with growth regulatory materials of the testa to promote germination of the seed.

While the effect of GA_3 may or may not have a part to play in the germination of this seed, it was shown that this substance could be used successfully to overcome the effect of any inhibitors causing epicotyl dormancy in the seedling.

LITERATURE CITED

AMEN, R. D. 1968. A model of seed dormancy. Bot. Rev. 34:1-31.

ANSELL, G. B. and J. N. HAWTHORNE. 1964. Phospholipids. Vol. 3. Chemistry, metabolism and function. Amsterdam, Elsevier Publishing Co. 439 p.

BARTON, L. V. 1936. Germination and seedling production in *Lilium* species. Contrib. Boyce Thompson Inst. 8:297-309.

BARTON, L. V. 1965. Dormancy in seeds imposed by the seed coat. *In* Encyclopedia of Plant Physiology. Ed. by W. Ruhland. Berlin, Springer-Verlag. Vol. 15(2):727-745.

BARTON, L. V. and C. CHANDLER. 1957. Physiological and morphological effects of gibberellic acid on epicotyl dormancy of tree peony. Contrib. Boyce Thompson Inst. 19:201-214.

BLACK, M. and P. F. WAREING. 1954. Photoperiodic control of germination in seed of birch (*Betula pubescens* Ehrh.). Nature 174:705.

BORTHWICK, H. A. and S. B. HENDRICKS. 1960. Photoperiodism in plants. Science 132:1223-1228.

BRADBEER, J. W. 1968. Studies in seed dormancy. IV. The role of endogenous inhibitors and gibberellin in the dormancy and germination of *Corylus avellana* L. seeds. Planta 78:266-276.

BURNS, R. M. 1967. Gibberellic acid stimulates germination of Sweetgum seed. Forest Sci. 13:438-439.

CLELAND, R. E. 1969. The gibberellins. *In* The Physiology of Plant Growth and Development. Ed. by M. B. Wilkins. New York, McGraw-Hill. 49-81.

CONN, E. E. and P. K. STUMPF. 1967. Outlines of Biochemistry. 2d ed. New York, John Wiley & Sons, Inc. 468 p.

CORNFORTH, J. W., B. V. MILBORROW and G. RYBACK. 1966. Identification and estimation of (+)-abscisin II ('Dormin') in plant extracts by spectropolarimetry. Nature 210:627-628.

CORNS, WM. G. 1960. Effects of gibberellin treatments on germination of various species of weed seeds. Can. J. Plant Sci. 40:47-51.

CORNS, WM. G. and R. J. SCHRAA. 1962. Dormancy and germination of seeds of Silverberry (*Elaeagnus commutata* Bernh.). Can. J. Bot. 40:1051-1055.

COSSINS, E. A. and H. BEEVERS. 1963. Ethanol metabolism in plant tissue. Plant Physiol. 38:375-380.

CURTIS, E. J. C. and J. E. CANTLON. 1968. Seed dormancy and germination in *Melampyrum lineare*. Amer. J. Bot. 55:26-32.

DALETSKAYA, T. V. 1964. The problem of the role of β-indoleacetic acid in seed dormancy. (Transl.) Akademiia Nauk SSSR Doklady (Botanical Sciences) 156:708-711.

DURZAN, D. J. and V. CHALUPA. 1968. Free sugars, amino acids, and soluble proteins in the embryo and female gametophyte of jack pine as related to climate at the seed source. Can. J. Bot. 46:417-428.

EVENARI, M. 1965. Light and seed dormancy. *In* Encyclopedia of Plant Physiology. Ed. by W. Ruhland. Berlin, Springer-Verlag. Vol. 15(2):804-847.

FINE, J. M. and L. V. BARTON. 1958. Biochemical studies of dormancy and after-ripening in seeds. I. Changes in free amino acid content. Contrib. Boyce Thompson Inst. 19:483-500.

FIRENZUOLI, A. M., P. VANNI, E. MASTRONUZZI, A. ZANOBINI and V. BACCARI. 1968. Enzymes of glyoxylate cycle in conifers. Plant Physiol. 43:1125-1128.

FLEMION, F. 1937. A rapid method for determining the viability of dormant seeds. Contrib. Boyce Thompson Inst. 9:339-351.

FOREST SERVICE. 1948. Woody-Plant Seed Manual. U.S.D.A. Misc. Pub. 654. 416 p.

GIERSBACH, J. 1937. Germination and seedling production of species of *Viburnum*. Contrib. Boyce Thompson Inst. 9:79-90.

GREEN, D. E. and K. KOPACZYK. 1966. The repeating units of membranes. Acta Biochimica Polonica 13:417-427.

HORROCKS, L. A. 1963. Thin-layer chromatography of brain phospholipids. J. Amer. Oil Chem. Soc. 40:235-236.

IKUMA, M. and K. V. THIMANN. 1964. Analysis of germination processes of lettuce by means of temperature and anaerobiosis. Plant Physiol. 39:756-767.

JACKSON, G. A. D. and J. B. BLUNDELL. 1965. Germination of *Rosa arvensis*. Nature 205:518-519.

JANSON, J. C. 1967. Adsorption phenomena on Sephadex. J. Chromatog. 28:12-20.

JARVIS, B. C., B. FRANKLAND and J. H. CHERRY. 1968a. Increased DNA template and RNA polymerase associated with the breaking of seed dormancy. Plant Physiol. 43:1734-1736.

JARVIS, B. C., B. FRANKLAND and J. H. CHERRY. 1968b. Increased nucleic-acid synthesis in relation to the breaking of dormancy of hazel seed by gibberellic acid. Planta 83:257-266.

KNOWLES, R. H. 1957. Studies on dormancy of seed of the American high-bush cranberry, *Viburnum trilobum* Marsh. M.Sc. Thesis. Univ. Alberta.

KNOWLES, R. H. and S. ZALIK. 1958. Effects of temperature treatment and of a native inhibitor on seed dormancy and of cotyledon removal on epicotyl growth in *Viburnum trilobum* Marsh. Can. Jour. Bot. 36:561-566.

KOCH, F. C. and T. L. McMEEKIN. 1924. A new direct nesslerization microkjeldahl method and a modification of the Ness-Folin reagent for ammonia. J.A.O.C.S. 46:2066-2069.

KOLLER, D., A. M. MAYER, A. POLJAKOFF-MAYBER and S. KLEIN. 1962. Seed germination. Ann. Rev. Plant Physiol. 13:437-464.

MAYER, A. M. and A. POLJAKOFF-MAYBER. 1963. The Germination of Seeds. New York, Pergamon Press, MacMillan. 236 p.

MER, C. L. 1968. What is an inhibitor? Z. Pflanzenphysiol. 59:415-419.

MITCHELL, J. W. and B. C. SMALE. 1963. Bioassay — plants. *In* Analytical Methods for Pesticides, Plant Growth Regulators and Food Additives. Ed. by G. Zweig. New York, Academic Press. Vol. 1:443-469.

MORHOLT, E., P. F. BRANDWEIN and A. JOSEPH. 1966. A Sourcebook for Biological Sciences. 2d ed. New York, Harcourt, Brace and World, Inc. 795 p.

NICHOLS, B. W. 1964. The separation of lipids by thin-layer chromatography. Lab. Pract. 13:299-305.

NORBERG, S. O. 1968. Studies in the production of auxins and other growth stimulating substances by Exobasidium. Symb. Bot. Upsal. XIX(3):1-117.

PAECH, K. and M. TRACEY (eds.). 1965. Modern Methods of Plant Analysis. Berlin, Springer-Verlag. Vol. 7:127.

RAGHAVAN, V. and J. G. TORREY. 1964. Effects of certain growth substances on the growth and morphogenesis of immature embryos of *Capsella* in culture. Plant Physiol. 39:691-699.

REDSHAW, E. S. 1968. Vernalization studies on cereals. Ph.D. Thesis. Univ. Alberta.

REDSHAW, E. S. and S. ZALIK. 1968. Changes in lipids of cereal seedlings during vernalization. Can. J. Biochem. 46:1093-1097.

ROBINSON, T. 1967. The Organic Constituents of Higher Plants. 2d ed. Minneapolis, Burgess Publishing Co. 319 p.

ST. ANGELO, A. J. and A. M. ALTSCHUL. 1964. Lipolysis and the free fatty acid pool in seedlings. Plant Physiol. 39:880-883.

SANE, P. V. 1968. Biochemical studies on a chlorophyll mutant of Gateway barley. Ph.D. Thesis. Univ. Alberta.

SCHRAMM, R. W. 1967. The part of organic acids in germination of seeds. Acta Soc. Bot. Pol. 36:39-55.

SCHUETTE, H. A. and J. A. KORTH. 1940. The seed oil of the highbush cranberry. Oil & Soap 17:265.

SCIFRES, C. J. and M. K. McCARTY. 1969. Some factors affecting germination and seedling growth of Scotch thistle. Univ. Nebraska College of Agric., Agric. Exptl. Res. Bull. 228:1-28.

SINGH, A. 1967. Plant Physiology. New York, Asia Publishing House. 615 p.

SKIPSKI, V. P., A. F. SMOLOWE, R. C. SULLIVAN and M. BARCLAY. 1965. Separation of lipid classes by thin-layer chromatography. Biochim. Biophys. Acta 106:386-396.

SPLITTSTOESSER, W. E. 1969a. Arginine metabolism by pumpkin seedlings. Separation of plant extracts by ion exchange resins. Plant & Cell Physiol. 10:87-94.

SPLITTSTOESSER, W. E. 1969b. The appearance of arginine and arginase in pumpkin cotyledons. Characterization of arginase. Phytochem. 8:753-758.

STEARNS, F. and J. OLSON. 1958. Interactions of photoperiod and temperature affecting seed germination in *Tsuga canadensis*. Amer. J. Bot. 45:53-58.

STOKES, P. 1953a. A physiological study of embryo development in *Heracleum sphondylium* L. III. The effect of temperature on metabolism. Ann. Bot. 17:157-173.

STOKES, P. 1953b. The stimulation of growth by low temperature in embryos of *Heracleum sphondylium* L. J. Exptl. Bot. 4:222-234.

STOKES, P. 1965. Temperature and seed dormancy. *In* Encyclopedia of Plant Physiology. Ed. by W. Ruhland. Berlin, Springer-Verlag. Vol. 15(2):746-803.

STRONG, F. M. 1958. Topics in Microbial Chemistry. New York, John Wiley and Sons. 166 p.

STUMPF, P. K. 1962. Lipid metabolism in higher plants. Nature 194: 1158-1160.

THORNTON, N. C. 1935. Factors influencing germination and development of dormancy in cocklebur seeds. Contrib. Boyce Thompson Inst. 7:477-496.

TOOLE, E. H., S. B. HENDRICKS, H. A. BORTHWICK and V. K. TOOLE. 1956. Physiology of seed germination. Ann. Rev. Plant Physiol. 7:299-324.

VAN OVERBEEK, J. 1966. Plant hormones and regulators. Science 152: 721-731.

VAN OVERBEEK, J., M. E. CONKLIN and A. F. BLAKESLEE. 1942. Cultivation *in vitro* of small *Datura* embryos. Amer. J. Bot. 29:472-477.

VARNER, J. E. 1965. Seed development and germination. *In* Plant Biochemistry. Ed. by J. Bonner and J. E. Varner. New York, Academic Press. 1054 p.

VILLIERS, T. A. and P. F. WAREING. 1965. The possible role of low temperature in breaking the dormancy of seeds of *Fraxinus excelsior* L. J. Exptl. Bot. 16:519-531.

WANG, D. 1968. Metabolism of amino acids and amides in germinating seeds. Contrib. Boyce Thompson Inst. 24:109-115.

WARD, G. M. and F. B. JOHNSTON (eds.). 1962. Chemical Methods of Plant Analysis. Pub. 1064. Ottawa, C.D.A. 59 p.

WAREING, P. F. 1966. Natural inhibitors as growth hormones. *In* Trends in Plant Morphogenesis. Ed. by E. G. Cutter. London, Longmans, Green and Co. 329 p.

ZIMMERMAN, D. C. and H. J. KOLSTERMAN. 1965. Lipid metabolism in germinating flaxseed. Amer. Oil Chem. Soc. Jour. 42:58-62.